REMARKS

Claims 1-11 and 14-23 are now in the case. Claims 27-31 have been cancelled. Claims 12, 13, 24 and 25 have been withdrawn as being drawn to a non-elected invention. Claims 2, 3, 6, 7, 10, 15, 19, and 22 have been amended to reflect the election of SEQ ID NO:12. No new matter has been added.

PRELIMINARY AMENDMENT AND SPECIFICATION

The Action notes the following informalities: The amendment to page 1 of the specification does not indicate the status of the parent application serial No. 09/890,323, as abandoned. Applicant is invited to amend the specification. Also, Applicant is required to delete all instances of embedded hyperlinks and/or other forms of browser-executable code.

Applicant has provided the requested amendments as is indicated above.

ELECTION/RESTRICTIONS

The Action requests Applicant to affirm the election of Group I, comprising claims 1-11, 14-23 and 26-31 drawn to an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:12.

Applicant affirms the election with traverse. Claims 12, 13, 24 and 25 are withdrawn as being drawn to a non-elected invention. Claims 2, 3, 6, 7, 10, 15, 19, and 22 have been amended to reflect the election of SEQ ID NO:12.

REJECTION UNDER 35 U.S.C. §101 and §112, First Paragraph

Claims 1-11, 14-23 and 26-31 stand rejected under 35 U.S.C. §101 and §112, first paragraph, on the basis that these claims allegedly lack patentable utility, and that one skilled in the art would therefore not know how to use the invention.

Applicant respectfully traverses this rejection. Merely to advance the claims towards allowance, claims 27-31 have been cancelled. Applicants reserve the right to pursue the cancelled matter in continuation applications.

Utility under 35 U.S.C. §101 is a minimal threshold issue that can be satisfied by a showing of any use that is "substantial," "credible," and "specific." (MPEP §2107). A small degree of utility is sufficient. Thus, as a matter of Patent Office practice, a specification that

provides disclosure of a utility that corresponds in scope to the subject matter sought to be patented and that is substantial, credible, and specific *must* be taken as sufficient to satisfy the utility requirement of 35 U.S.C. §101 for the entire claimed subject matter unless there is reason for one skilled in the art to question the objective truth of the statement of utility.

The specification states that SVPH-1 was isolated from human testis, see page 14, line 27. At page 15, lines 1-3, the specification provides that SVPH1a was isolated from a human testis library. Example 4 (bridging pages 48-49) describes Northern Blot analysis determining that SVPH-1 is specifically expressed in testis. The Action readily acknowledges that the mRNA transcripts encoding the SVPH1a metalloprotease can be detected in human testicular tissue.

SVPH1 and its splice variant SVPH1a are testis-specific proteins and because of this tissue specificity, SVPH1a polypeptides and fragments thereof, corresponding polynucleotides and antibodies directed to the polypeptide are useful in identifying and/or isolating testes cells. This use is credible, as Applicant has demonstrated in Example 4 on pages 48-49 of the specification that SVPH1 expression was detected only in testis tissue and its splice variant, SVPH1a was isolated from testis tissue. This use is specific, because not all polypeptides, antibodies, or polynucleotides will bind to testes cells, and certainly not all polypeptides, antibodies, or polynucleotides will bind to testes cells with the specificity of polypeptides or fragments of SEQ ID NO:12 (SVPH1a). This use is also substantial in that there are "real world" uses for compounds that specifically bind to testes cells: the purification of such cells, and in diagnosis of testes-related disorders that alter the location, number, or morphology of testes cells, such as cryptorchidism (failure of one or both of the testes to descend), male infertility, or testicular cancer. Tissue specific proteins are also valued for their use as delivery agents as carriers of detectable (diagnostic) and therapeutic agents that may be attached to a tissue specific molecule. The agents are useful for delivering agents specifically to the desired target due to the tissue specificity of the delivery compound (see page 37, lines 10-33). Such uses include tissue specific localization of toxins or cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetirc or flurometric reaction and the like (see page 37, lines 15-16) for tissue specific diagnostic and therapeutic purposes, such as diagnosis and treatment of testicular cancer.

Applicant submits that uses of polypeptides and polynucleotides for purposes other than those associated with a specific activity of a protein are known and have repeatedly been

used by those of skill in the art. Applicant submits that the uses described above for SVPH1a polypeptides of SEQ ID NO:12 and fragments of such, are credible and specific. The use of such tissue specific proteins would be readily apparent to one of skill in the art and immediate application of such tissue specific polypeptides, for uses such as those described above, would be recognized. The "Revised Interim Utility Guidelines Training Materials", available at the USPTO web site, elucidates the process by which Examiners and Applicants should analyze the utility aspect of an invention under 35 U.S.C. §101. For example, with respect to "credible utility" the guidelines explain that,

> Credibility as used in this context refers to the reliability of the statement based on the logic and facts that are offered by the applicant to support the assertion of utility. A *credible* utility is assessed from the standpoint of whether a person of ordinary skill in the art would accept that the recited or disclosed invention is currently available for such use. (Emphasis in original).

For the present invention to lack credible utility the guidelines state that the invention must lack an acceptable use based upon the standpoint of one of ordinary skill in the art. The Action does not provide a reason why one of ordinary skill in the art would not accept the SVPH1a polypeptides of the invention as being useful for testis tissue-specific purposes. In fact, those of skill in the art often use antibodies to a protein or polynucleotides encoding a specific protein to identify tissues as well as diseases and to measure expression and changes in expression relative to other genes or samples. Accordingly, Applicant submits that one of skill in the art would recognize the use of the SVPH1a polypeptides as being credible.

In addition, Applicant submits that not only is the use of SVPH1a polypeptides "credible," but the use is also "specific." The guidelines suggest that a specific utility would include, for example, a polynucleotide that can be used as a chromosomal marker where it specifically binds to a DNA target. The guidelines explain that,

> For example, a claim to a polynucleotide whose use is disclosed simply as a . . . "chromosome marker" would not be considered to be specific *in the absence* of a disclosure of a specific DNA target. (Emphasis added).

By analogy, Applicant submits that the SVPH1a polypeptide have a credible and specific utility as, for example, tissue markers having a specific identified target, *e.g.*, testis cells and tissue. Such a use satisfies both the credible and specific utilities. As discussed above, the specification teaches that SVPH1a polypeptides identify with a specific tissue target (*e.g.*,

testis). The guidelines indicate, with respect to chromosomal markers, that in "the absence" of a specific target a polynucleotide would lack specific utility. Applicant has provided a specific target for tissue identification (*e.g.*, testis) and thus Applicant submits that the SVPH1a polypeptide of the invention has a specific utility. Accordingly, the SVPH1a polypeptides and fragments thereof provide a "substantial," "credible," and "specific" utility in accordance with 35 U.S.C. §101 and thus satisfy the USPTO's guidelines on utility.

The Office Action also alleges that the use of the SVPH1a polypeptides "for further research to determine, *e.g.*, its specific biological role, thus identifying or confirming a "real world" context for its use, cannot be considered to be a "substantial utility." Applicant respectfully disagrees and submits that U.S. Patents issue regularly that feature reagents used in research. Just a few notable examples are Mullis *et al.* U.S. 4,687,195; Mullis *et al.* U.S. 4,683,202; and Gelfand *et al.* U.S. 4,889,818, featuring PCR reagents, kits and techniques, which are used as investigatory tools to amplify DNA. The rejection in this case is equivalent to a decision that the PCR technique is not useful under the patent statute because no utility has been shown for the DNA it can amplify. The U.S. Patent and Trademark Office recognizes the patentable utility of reagents and methodologies used in such investigations. To permit some patents on research tools while denying others is arbitrary and capricious.

Therefore, for at least the reasons presented above, Applicant has asserted in the specification a specific, substantial, and credible use for compositions of matter of the invention, and withdrawal of the rejection of claims 1-11, 14-23 and 26-31 under 35 U.S.C. §101 and §112, first paragraph, is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, First Paragraph (written description)

Claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, First Paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the invention was filed, had possession of the claimed invention.

Applicants respectfully traverse these grounds for rejection. As discussed above, claims 26-31 have been cancelled. It was well known to those of ordinary skill in the art that the disintegrin domains of certain ADAM polypeptides such as fertilin beta (ADAM2), present in sperm, interact with integrin molecules on eggs; peptides from the disintegrin domain of fertilin beta (ADAM2) specifically inhibit egg-sperm fusion, presumably by

blocking the endogenous ADAM2 disintegrin from binding to integrin, see page 2, lines 20-21 of Applicant's specification and Myles et al., "Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion" *Proc Natl Acad Sci USA* 91: 4195-4918, 1994 (a copy of which is provided herein). Furthermore, disintegrin domain peptides from an ADAM polypeptide specifically expressed in the testes of Xenopus, xMDC16 (ADAM16), was also shown to inhibit fertilization of Xenopus oocytes (Shilling et al., "Identification of Metalloprotease/Disintegrins in *Xenopus laevis* Testis with a Potential Role in Fertilization" *Dev Biol* 186: 155-164, 1997, a copy of which is provided herein). These testis-specific ADAM proteins involved in fertilization and/or spermatogenesis, and ADAM12 (meltrin alpha) involved in a cell-cell fusion process analogous to fertilization: fusion of myoblasts into muscle cells (specification, page 2, lines 21-22), were known in the art at the time of Applicant's filing. As described above, SVPH1a was detected only in testis tissue. Given the knowledge in the art that different testis associated ADAM disintegrin domain polypeptides were involved in spermatogenesis and/or fertilization and that molecules comprising amino acid sequences from the disintegrin domains of several different ADAM polypeptides could be used in ways to inhibit fertilization, taken in combination with the teaching in Applicant's specification regarding the testis tissue specificity of SVPH1a, one of ordinary skill in the art would have understood that a disintegrin activity for a testis-specific disintegrin domain-containing protein would be an activity affecting (inhibiting) spermatogenesis and/or fertilization.

Therefore, at least the above reasons stated above, Applicant believes that the bases for the rejection of claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, First Paragraph (written description), have been overcome or have been eliminated; withdrawal of the rejection of these claims is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, First Paragraph (enablement)

Claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, First Paragraph, because the specification, while being enabling for the preparation of a polypeptide having the amino acid sequence of the elected SEQ ID NO:12 having a disintegrin activity, does not reasonably enable preparation of amino acid sequences having disintegrin activity that diverge from the amino acid sequence of SEQ ID NO:12 by unlimited amino acid substitutions, deletions and insertions, or combinations thereof anywhere within SEQ ID NO:12.

Applicants respectfully traverse these grounds for rejection. As discussed above, claims 26-31 have been cancelled. At the time of filing of Applicant's application, one of skill in the art was aware of the structure of a disintegrin domain, particularly the conserved structural features of the disintegrin domain within ADAM polypeptide such as fertilin-alpha, and the relationship between these features and the functions of these domains (please see Jia et al., *J. Biol. Chem.* 272: 13094-13102, 1997, a copy of which is provided). Figure 1 of Jia et al., shows the sequence similarity between disintegrin domains such as those of murine fertilin-alpha and fertilin-beta, and snake venom disintegrin domains such as that of atrolysin A. Of particular note in Figure 1 is the conservation of the overall number and arrangement of cysteine residues between disintegrin domains, and particularly between the eight disintegrin domain amino acid sequences in the lower part of Figure 1. Attached is an amino acid sequence alignment showing the amino acid sequence similarity between the disintegrin domain region of SVPH1a polypeptide (amino acid residues 389 through 491 of SEQ ID NO:12, see page 8, lines 12-13 of the specification) and the disintegrin domains of fertilin-alpha, fertilin-beta, and atrolysin A. As noted in the specification, all ADAMs contain a disintegrin domain that is approximately 80 amino acids in length with 15 highly conserved Cys residues (see page 2, lines 8-9). The SVPH1a polypeptide has the 15 conserved cysteine residues present in these other disintegrins. One of skill in the art would know to design the boundaries of a fragment of SEQ ID NO:12 having disintegrin activity to include these residues important to disintegrin function to make and use Applicant's claimed invention.

Therefore, at least the above reasons stated above, Applicant believes that the bases for the rejection of claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, First Paragraph (enablement), have been overcome or have been eliminated; withdrawal of the rejection of these claims is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, Second Paragraph

Claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, Second Paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Applicant respectfully traverses these grounds for rejection. As discussed above, claims 27-31 have been cancelled. The Action states that claims 1, 6, 15 and 27 are indefinite in stating "having disintegrin activity". As discussed above, given the knowledge in the art

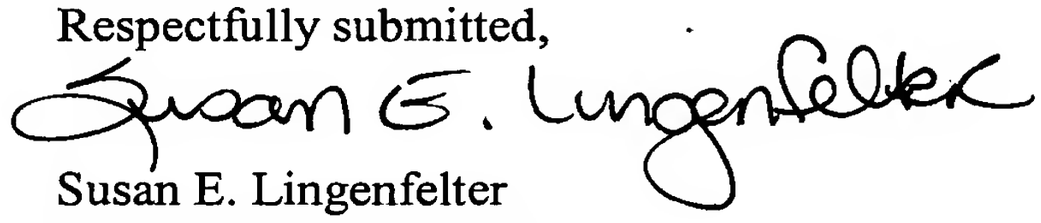
that different testis-associated ADAM polypeptides were involved in spermatogenesis and/or fertilization and that molecules comprising amino acid sequences from the disintegrin domains of several different ADAM polypeptides could be used in ways to inhibit fertilization, taken in combination with the teaching in Applicant's specification regarding the testis tissue specificity of SVPH1a, one of ordinary skill in the art would have understood that a disintegrin activity for a testis-specific disintegrin domain-containing protein would be an activity affecting (inhibiting) spermatogenesis and/or fertilization.

Therefore, at least the above reasons stated above, Applicant believes that the bases for the rejection of claims 1-11, 14-23, and 26-31 stand rejected under 35 U.S.C. §112, Second Paragraph, have been overcome or have been eliminated; withdrawal of the rejection of these claims is respectfully requested.

CONCLUSION

Applicants submit that the presented claims are in condition for allowance. A favorable action is earnestly requested. Applicants' attorney invites the Examiner to call her at the number below if any issue remains outstanding.

Respectfully submitted,



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Seattle, WA 98119
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gl143301 12/19/05

Plurality: 2.00 Threshold: 4 AveWeight 1.00 AveMatch 2.78 AvMisMatch -2.25

Consensus Sequence

Symbol comparison table: blosum62.cmp CompCheck: 1102

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GapLengthWeight: 2

PileUp MSF: 103 Type: P December 15, 2005 15:57 Check: 1647 ..

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Name: SVPH1a	Len: 103	Check: 8858	Weight:	1.00
Name: FERT_B_MUS	Len: 103	Check: 3273	Weight:	1.00
Name: ATROLYSIN_A	Len: 103	Check: 9880	Weight:	1.00

//

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             1                                                  50
    SVPH1a VHTKDIFNVK RCGNGVVEEG EECDCGPLKH CAK..DPCCL S.NCTLTDGS
 FERT_A_MUS ~~~~~~~~~~ ~CGNGVVEDL EECDCG..SD CDS..HPCCS P.TCTLKEGA
 FERT_B_MUS ~~~~~~~~~~ ~CGNGEVEED EICDCGK.KG CAEMPPPCCN PDTCKLSDGS
ATROLYSIN_A ~~~~~~~SPP VCGNELLEVG EECDCGSPRT C...RDPCCD AATCKLHSWV
  Consensus ---------- -CGNGVVEEG EECDCG--K- CA---DPCC- P-TC-L-DGS
                        (1)        (2)(3)    (4)    (5)(6)  (7)
             51                                                 100
    SVPH1a TCAFGLCCKD CKFLPSGKVC RKEVNECDLP EWCNGTSHKC PDDFYVEDGI
 FERT_A_MUS GCSEGLCCYN CTFKKKGSLC RPAEDVCDLP EYCDGSTQEC PANSIMQDG~
 FERT_B_MUS ECSSGICCNS CKLKKKGEVC RLAQDECDVT EYCNGTSEVC .EDFFVQNG~
ATROLYSIN_A ECESGECCQQ CKFTSAGNVC RPARSECDIA ESCTGQSADC PTDDFHRNG~
  Consensus ECSSGLCC-- CKFKKKG-VC RPA-DECDLP EYCNGTS--C P-DFFVQ-G-
             (8)  (9)(10) (11)      (12)      (13)    (14)  (15)
             101
    SVPH1a PCK
 FERT_A_MUS ~~~
 FERT_B_MUS ~~~
ATROLYSIN_A ~~~
  Consensus ---
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Cell Biology

Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion

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ABSTRACT **Fertilization and certain later stages in mammalian embryonic development require fusion between membranes of individual cells. The mechanism of eukaryotic cell–cell fusion is unknown, and no surface molecules required for this process have been unequivocally identified. The role of the sperm surface protein fertilin in sperm–egg fusion was tested by using peptide analogues of a potential integrin binding site in the fertilin β subunit. Peptide analogues that include a TDE sequence from the disintegrin region of fertilin β are able to bind to the egg plasma membrane and strongly inhibit sperm–egg fusion. These results show that the disintegrin domain of fertilin β binds to the egg plasma membrane and that this binding is required for membrane fusion.**

Membrane fusion is important in many different cellular functions. The majority of membrane fusion events involve the membranes within a single cell, and these processes are being intensively studied. Much less is known about the molecular mechanism of fusion between the plasma membranes of two cells. Our investigations have focused on the fusion between the sperm and the egg plasma membranes, a key event in development.

Our initial antibody inhibition studies identified a protein on the guinea pig sperm surface called fertilin that is involved in sperm–egg membrane fusion (1), but the exact role of fertilin remained unsolved. Fertilin (originally named PH-30 because of its localization to the posterior head domain of the sperm) is a heterodimeric protein. Analysis of the cDNA sequence of guinea pig fertilin α and β subunits revealed that the N-terminal region of the mature β subunit has high homology with a family of integrin ligands, the disintegrins, and that the α subunit contains a putative fusion peptide, analogous to the fusion peptides of viruses (2). The presence of the disintegrin sequence led to the hypothesis that fertilin is a novel type of cell surface integrin ligand in that the disintegrin domain of fertilin β might bind to an egg integrin and this binding might be required for sperm–egg fusion. To test this hypothesis we examined the ability of peptide analogues derived from the putative fertilin β binding site to bind to the egg plasma membrane and to block sperm–egg fusion. The results of this study indicate that sperm bind to the egg plasma membrane through the disintegrin domain of fertilin β and that this binding step is required for sperm–egg fusion.

MATERIALS AND METHODS

Peptides. Peptides were a generous gift of Christopher Turck (University of California, San Francisco) or were prepared by the Yale University Peptide Synthesis Facility (New Haven, CT).

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Covasphere Binding. Violet MX Covaspheres, 0.8-μm (Duke Scientific, Palo Alto, CA), were conjugated with CSTDEC or CTESDC peptide at 1 mg/ml (200 μg of peptide per 50 μl of Covaspheres), following the manufacturer's instructions. Unreacted sites were blocked with 1% glycine. Covaspheres (10 μl) were added to oocytes in a 100-μl drop of modified Tyrode's solution (mT), mixed well, and incubated under mineral oil for 3 h at 37°C with 95% air/5% CO_2. Oocytes were washed free of excess beads by pipetting through five 50-μl drops of mT and were mounted on slides in 25 μl of mT, compressed slightly with a coverslip, and photographed using a 345/425-nm filter set, and micrographs were scored for the total number of fluorescent beads bound per half-oocyte. Because few Covaspheres bound over the cortical granule-free region of the oocytes, this region was bisected for counting.

***In Vitro* Fertilization Assays.** Guinea pig oocytes were collected from ovaries and matured, and the zonae were removed as previously described (1). For zona-intact oocytes, matured oocytes were briefly treated with 0.25% hyaluronidase (Sigma) to remove the cumulus. Oocytes were washed after enzymatic treatment by pipetting through four 400-μl drops of mT and then put into a 100-μl drop with or without peptide, followed by incubation at 37°C in 95% air/5% CO_2 for 30 min. Sperm were capacitated, the acrosome reaction was induced, and fusion assays were carried out as previously described (1, 3). Sperm concentrations were in the range $1–5 \times 10^4$ per ml for zona-free eggs and $2.5–5 \times 10^5$ per ml for zona-intact eggs. Fusion was scored by the presence of swollen sperm heads after acetolacmoid staining (4). We determined that this method gave results equivalent to those obtained by following a protocol in which fusion was scored by preloading the eggs (5) with the fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Polyscience), which stains the nuclei of fused sperm.

RESULTS AND DISCUSSION

Comparison of the deduced amino acid sequence of fertilin β to the sequence of other members of the disintegrin family was used to design test peptides (Fig. 1). Snake venom small peptides, the first identified members of the disintegrin family, bind to the platelet integrin GPIIb-IIIa ($\alpha_{IIb}\beta_3$) and inhibit platelet aggregation (18, 19). The sequences of most of these small disintegrins include the tripeptide RGD as part of the binding site (kistrin, bitistatin, echistatin, barbourin; Fig. 1*a*) (18–20). The disintegrin family also includes larger proteins from snake venom [jararhagin (10), HR1B (11), and

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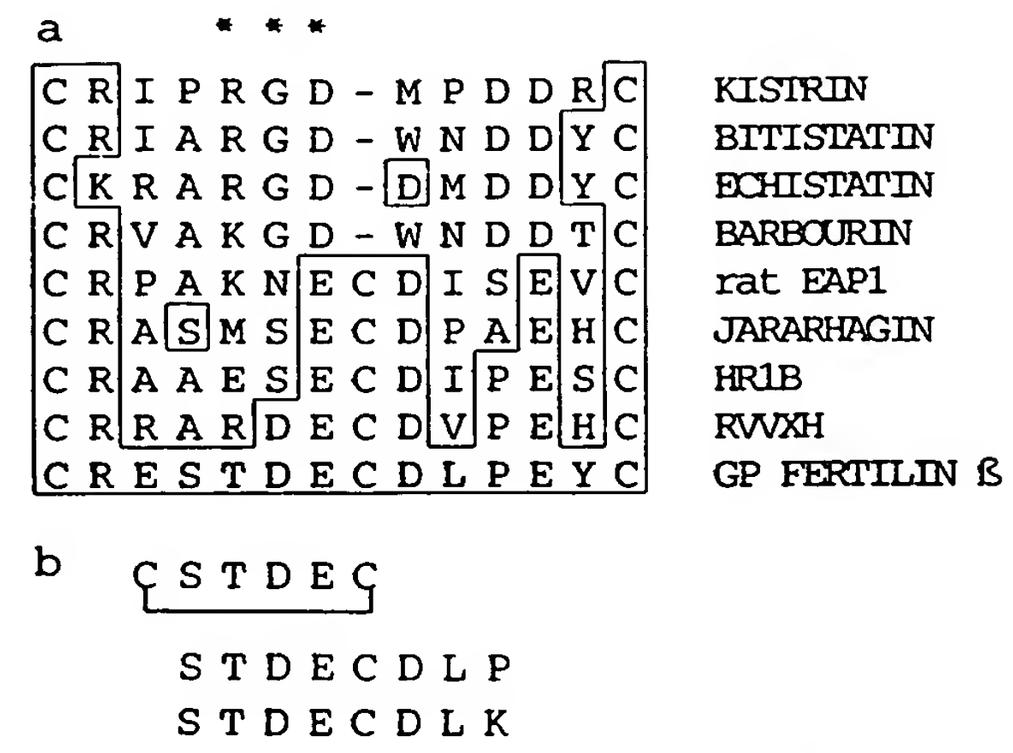

FIG. 1. Sequence comparison of known binding regions of small disintegrins with putative binding regions of disintegrin domains of guinea pig fertilin β and of large snake venom proteins (*a*) and fertilin β peptides tested in this study (*b*). Shown are relevant sequences from small disintegrins (48–83 residues) isolated from snake venoms [kistrin (6), bitistatin (7), echistatin (8), barbourin (9)] and larger snake venom proteins [jararhagin (10), HR1B (11), RVV-X heavy chain (12)] and the surface protein rat EAP1 (13) that share with fertilin β the disintegrin domain and two other domains (14). The sequences shown are the 13 amino acids that form the RGD-containing loop of kistrin (15) and echistatin (16, 17) and the corresponding 13 or 14 amino acids of the other disintegrin domains. The whole disintegrin domain of fertilin β is the N-terminal 90 amino acids of mature fertilin β (2). The percentage of identical amino acids in the whole disintegrin domain compared with fertilin β is 44% for kistrin; 54% for bitistatin; 36% for echistatin; 47% for barbourin; 54% for jararhagin; 54% for HR1B; 58% for the heavy chain of RVV-X, RVVXH; and 44% for rat EAP1. The peptides tested are shown in *b*, aligned with the sequence of the putative binding site shown in *a*.

RVV-X (12)] and a few cell surface proteins of unknown functions—for example, the mammalian surface protein EAP1 (13). Like fertilin β, these other proteins have alternative amino acids aligned with the RGD sequence. These alternative amino acids are followed by a cysteine not found in the smaller snake venom disintegrins (Fig. 1*a*). Fertilin β has a substitution of TDE in place of the RGD tripeptide (Fig. 1*a*). Two peptides were chosen to be tested on the basis of the amino acid sequence in this region: CSTDEC and STDECDLP (Fig. 1*b*). In addition, a variant of STDECDLP with a K substituted for the final P was tested (this variant peptide was originally synthesized for a separate study).

The CSTDEC peptide was cyclized prior to testing because a cyclized peptide might better mimic the native binding site.

Table 1. Peptide-coated Covasphere binding to zona-free eggs

Peptide	No. of eggs	No. of expts.	No. of Covaspheres bound per egg: Mean	No. of Covaspheres bound per egg: Mean − background
None	49	4	34 ± 3	—
CTESDC	102	8	42 ± 25	8
CSTDEC	102	8	86 ± 23	52

The number of peptide-coated Covaspheres bound was compared with the number of control beads bound (with no peptide). Results are mean ± SD. The confidence level for a significant difference between CSTDEC and no peptide is greater than 95%. The control scrambled peptide (CTESDC) showed no significant difference when compared with no peptide ($P = 0.04$).

Structural studies of the snake venom small disintegrins have demonstrated that the RGD tripeptide is located at the tip of a flexible hairpin loop created by disulfide bridges (15–17, 21) and that the binding activity of peptides to integrins is greater when the loop conformation is maintained (20). The additional cysteine (TDEC) that occurs in the potential binding site of the disintegrin domain of fertilin β and the larger snake venom proteins (Fig. 1*a*) could be free or disulfide bridged. The CSTDEC peptide was cyclized by oxidation, thereby mimicking either a loop conformation or disulfide bonding of the TDEC cysteine.

To determine if cyclized CSTDEC bound to the egg plasma membrane, we tested if CSTDEC-coated fluorescent Covaspheres would bind to the plasma membrane of zona-free eggs. Binding was compared to Covaspheres that were coated with a control cyclized peptide (CTESDC), containing the same amino acids but in a rearranged (scrambled) order. Covaspheres conjugated to the CSTDEC peptide bound to eggs at a level 6.5-fold higher than Covaspheres conjugated to the control scrambled peptide (Table 1). The finding that a peptide from the predicted binding site of fertilin β binds to the egg suggests that sperm can bind to the egg through fertilin β.

Because the PH-30 monoclonal antibody recognizes the β subunit of fertilin (22) and inhibits sperm–egg fusion (1), fertilin β-mediated binding would be expected to lead to fusion. To focus our experiments exclusively on physiologically competent sperm that bind to and then fuse with the egg, we tested the ability of TDE-containing peptides to inhibit sperm–egg fusion. Both the percentage of eggs fused with at least one sperm (fertilization rate) and the the mean number of sperm fused per egg (fertilization index) were scored. The control peptides tested were scrambled versions of both test peptides, with the same amino acids but in a

Table 2. Inhibition of sperm fusion with zona-intact eggs

Peptide	No. of eggs	No. of expts.	% of eggs fused (FR)	% inhibition of fusion as measured by FR	Mean no. of sperm fused per egg (FI)	% inhibition of fusion as measured by FI
None	195	16	83 ± 16	—	1.06 ± 0.32	—
CSTDEC	73	6	11 ± 14	87	0.11 ± 0.14	90
STDECDLP	47	4	16 ± 8	81	0.20 ± 0.10	81
STDECDLK	55	4	2 ± 4	98	0.02 ± 0.04	98
CTESDC	25	3	72 ± 15	13	0.80 ± 0.02	25
PDCTESDL	40	4	75 ± 17	10	0.90 ± 0.13	15
GRGES	35	3	68 ± 32	18	0.91 ± 0.27	14

TDE-containing peptides tested were cyclized CSTDEC and linear peptides STDECDLP and STDECDLK. Control peptides were either scrambled versions of test peptides (cyclized CTESDC and linear PDCTESDL) or an irrelevant peptide (GRGES). All peptides were tested at 500 μM. Results are mean ± SD. Confidence levels for a significant difference between all TDE-containing peptides and all controls, including no-peptide controls, are ≥95% ($P < 0.0001$, for both the fertilization rate and the fertilization index). Control peptides showed no significant difference when compared with no-peptide controls; P values for fertilization rate and fertilization index, respectively, were as follows: CTESDC, $P = 0.29$ and 0.19; PDCTESDL, $P = 0.38$ and 0.35; GRGES, $P = 0.22$ and 0.46.

Table 3. Inhibition of sperm fusion with zona-free eggs

Peptide	No. of eggs	No. of expts.	% of eggs fused (FR)	% inhibition of fusion as measured by FR	Mean no. of sperm fused per egg (FI)	% inhibition of fusion as measured by FI
None	165	13	74 ± 16	—	1.55 ± 0.49	—
CSTDEC	45	4	11 ± 10	85	0.13 ± 0.11	92
STDECDLK*	44	4	16 ± 14	78	0.23 ± 0.25	85
CTESDC	84	7	77 ± 17	0	2.10 ± 0.67	0
GRGES	35	3	78 ± 20	0	2.40 ± 2.5	0

Peptides are the same as those used in Table 2. All peptides were tested at 500 μM, except where noted. Results are mean ± SD. Confidence levels for a significant difference between all TDE-containing peptides and no peptide controls are ≥95% ($P < 0.0001$, for both fertilization rate and the fertilization index). Control peptides did not inhibit fusion.
*Two of these four experiments were carried out at a peptide concentration of 250 μM.

rearranged order, and an irrelevant peptide, GRGES. In sperm–egg fusion assays we tested both zona-intact and zona-free eggs.

The experiments with zona-intact eggs provide a test of the inhibitory activity of peptides on eggs that have not been treated with protease to remove the zona, a treatment that could alter the egg plasma membrane (23, 24). When zona-intact eggs were incubated with peptide prior to incubation with sperm, fusion of sperm with eggs was inhibited 81–98% in both the fertilization rate and the fertilization index in comparison with eggs incubated in the absence of peptide (Table 2). Control peptides inhibited at a much lower level (10–25%).

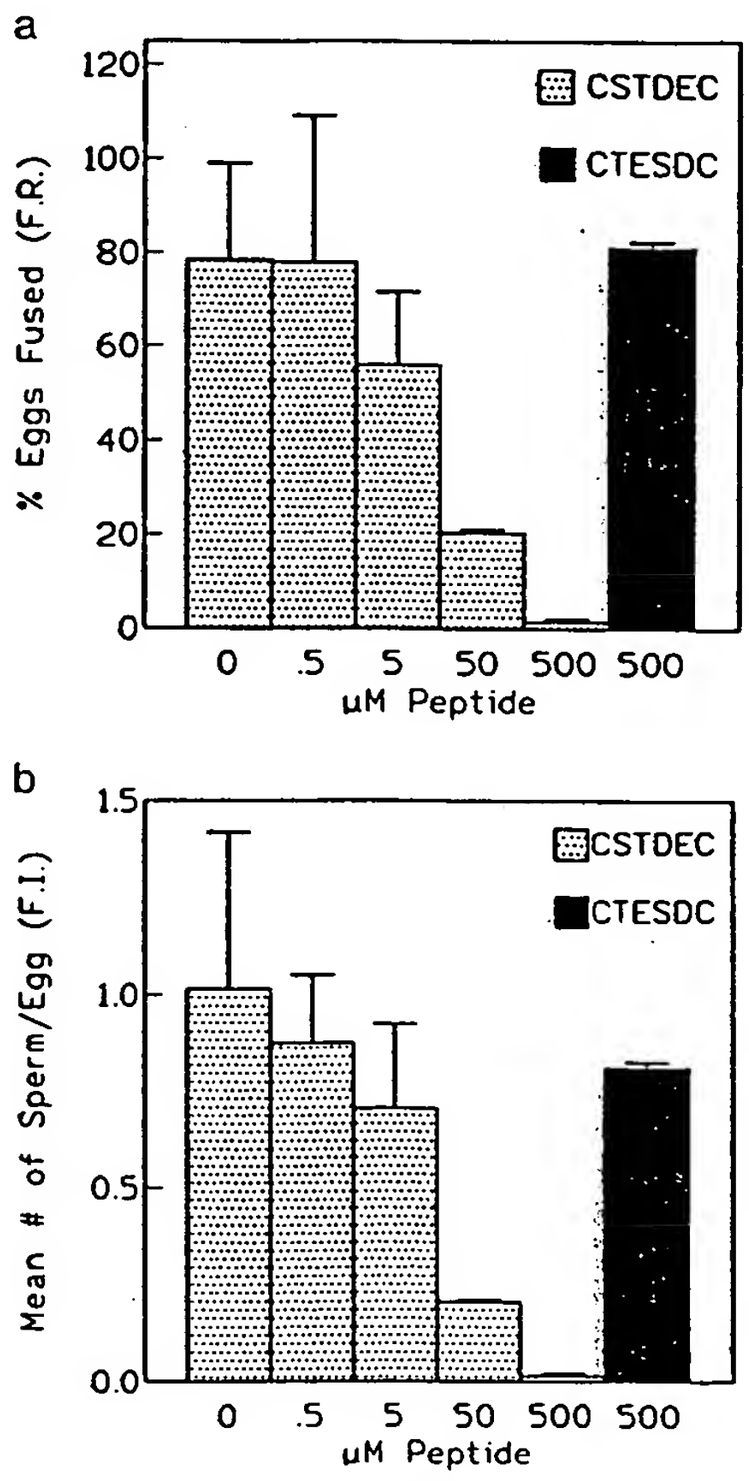

FIG. 2. Dose-dependent lowering of fertilization rate (*a*) and fertilization index (*b*) of zona-intact oocytes with the CSTDEC peptide. The results are the mean of two experiments for each peptide concentration from 0.5 to 500 μM, with the total number of eggs being 25 to 34 per peptide concentration. Inhibition at 500 μM peptide is taken as maximal and is close to 100%. Error bars represent SEM.

The experiments with zona-free eggs rule out the possibility of peptide inhibiting at the level of sperm adhesion to, or penetration through, the zona. In this case the TDE-containing peptides also strongly inhibited fusion as measured by the fertilization rate and the fertilization index (Table 3). The decrease of the fertilization rate caused by TDE peptides was 78–85% and the decrease of the fertilization index was 85–92%, when compared with eggs where no peptide was present. Control peptides caused no decrease in either the fertilization rate or the fertilization index. We do not know why we observed no effect of control peptides on either the fertilization rate or index in fusions with zona-free eggs, while there was a low inhibition in fusions with zona-intact eggs. There could be a low-level nonspecific inhibition by peptides of sperm–zona binding or penetration. This low-level inhibition is not observed in all experiments with zona-intact eggs (Fig. 2*a*).

The lowering of both the fertilization rate and the fertilization index of zona-intact eggs by CSTDEC was dose dependent. Half-maximal inhibition was between 5 and 50 μM (Fig. 2). This concentration is comparable to that required for inhibition of ligand–integrin GPIIb-IIIa binding by short RGD peptides that also inhibit in the micromolar range (18).

These experiments provide direct evidence for the role of the predicted fertilin β binding sequence (TDE) in sperm–egg binding and fusion. The results indicate that sperm fertilin binds to the egg plasma membrane by a mechanism that leads to sperm–egg fusion. In analogy to viral fusion proteins, fertilin β binding could result in a conformational change leading to the exposure of a hydrophobic fusion peptide in fertilin α (2) that could then promote membrane fusion (25).

The binding of fertilin β through its disintegrin domain is consistent with the hypothesis that the egg surface receptor for fertilin is an integrin. Since additional cell surface proteins with disintegrin domains have been reported (13, 26), fertilin may be a representative of an additional class of cell surface integrin ligands. Recent work has demonstrated the presence of several integrins on the surface of mammalian oocytes (24, 27). Inhibition of sperm fusion with hamster eggs has been observed with RGD-containing peptides (28) that may bind to one or more integrins, possibly competing with the binding of fertilin β on hamster sperm. The RGD sequence is not specific for a unique integrin, as is, for example, the KGD sequence of barbourin (9), and RGD-containing peptides can inhibit non-RGD ligand binding to integrins (29–31). Sperm binding to an egg integrin would mean that a potential pathway for sperm to signal the initiation of development (egg activation) would be through integrin-initiated signaling (32).

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Identification of Metalloprotease/Disintegrins in *Xenopus laevis* Testis with a Potential Role in Fertilization

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Proteins containing a membrane-anchored metalloprotease domain, a disintegrin domain, and a cysteine-rich region (MDC proteins) are thought to play an important role in mammalian fertilization, as well as in somatic cell–cell interactions. We have identified PCR sequence tags encoding the disintegrin domain of five distinct MDC proteins from *Xenopus laevis* testis cDNA. Four of these sequence tags (xMDC9, xMDC11.1, xMDC11.2, and xMDC13) showed strong similarity to known mammalian MDC proteins, whereas the fifth (xMDC16) apparently represents a novel family member. Northern blot analysis revealed that the mRNA for xMDC16 was only expressed in testis, and not in heart, muscle, liver, ovaries, or eggs, whereas the mRNAs corresponding to the four other PCR products were expressed in testis and in some or all somatic tissues tested. The xMDC16 protein sequence, as predicted from the full-length cDNA, contains a metalloprotease domain with the active-site sequence HEXXH, a disintegrin domain, a cysteine-rich region, an EGF repeat, a transmembrane domain, and a short cytoplasmic tail. To study a potential role for these xMDC proteins in fertilization, peptides corresponding to the predicted integrin-binding domain of each protein were tested for their ability to inhibit *X. laevis* fertilization. Cyclic and linear xMDC16 peptides inhibited fertilization in a concentration-dependent manner, whereas xMDC16 peptides that were scrambled or had certain amino acid replacements in the predicted integrin-binding domain did not affect fertilization. Cyclic and linear xMDC9 peptides and linear xMDC13 peptides also inhibited fertilization similarly to xMDC16 peptides, whereas peptides corresponding to the predicted integrin-binding site of xMDC11.1 and xMDC11.2 did not. These results are discussed in the context of a model in which multiple MDC protein–receptor interactions are necessary for fertilization to occur. © 1997 Academic Press

INTRODUCTION

During the past few years there have been several advances in our understanding of the molecular mechanism of vertebrate fertilization. In guinea pigs and mice, a sperm protein called fertilin (previously referred to as PH-30) is thought to be involved in sperm–egg membrane binding and perhaps also fusion (Almeida *et al.*, 1995; Blobel *et al.*, 1990, 1992; Blobel and White, 1992; Evans *et al.*, 1995; Myles, 1993; Myles *et al.*, 1994; Primakoff *et al.*, 1987). The involvement of fertilin in these events is supported by the following results: (i) One monoclonal antibody against fertilin blocks sperm–egg fusion, whereas a second one does not; (ii) fertilin is localized to the posterior sperm head, the region where fusion with the egg occurs (Primakoff *et al.*, 1987); and (iii) peptides that correspond to the predicted integrin-binding domain of fertilin (Blobel *et al.*, 1992) effectively inhibit guinea pig fertilization (Myles *et al.*, 1994). These results led to the hypothesis that an interaction between the sperm MDC protein fertilin (an MDC protein contains a membrane-anchored metalloprotease domain, a disintegrin domain, and a cysteine-rich region) and an integrin on the egg is a critical event in vertebrate fertilization. Indeed, several integrins are present on vertebrate eggs, and

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antibodies against the $\alpha 6\beta 1$ integrin block fertilization in the mouse (Almeida *et al.*, 1995). In addition to integrins, other yet to be identified proteins may function as cell-surface egg-receptors for sperm (for review see Foltz and Shilling, 1993; Myles, 1993; Snell and White, 1996). In sea urchin, for example, a membrane-associated egg receptor for sperm which is related to the hsp70 family of heat shock proteins has been identified (Foltz and Lennarz, 1990; Foltz *et al.*, 1993). In frog, starfish, and mouse, introduction of exogenous neurotransmitter and growth factor receptors into eggs and treatment with the appropriate ligand results in egg activation, suggesting that yet to be identified endogenous egg surface receptors and their ligands may be involved in egg activation via similar signaling pathways (Kline *et al.*, 1988; Moore *et al.*, 1993; Shilling *et al.*, 1994; Yim *et al.*, 1994).

The guinea pig sperm protein fertilin was the first recognized member of a family of membrane-anchored glycoproteins referred to as MDC proteins, which are related to soluble snake venom integrin ligands termed disintegrins (Weskamp and Blobel, 1994) and to snake venom metalloproteases (Bjarnason and Fox, 1995; Fox and Bjarnason, 1995; Rawlings and Barret, 1995). MDC proteins have also been referred to as ADAMs (proteins containing a disintegrin and metalloprotease domain; Wolfsberg and White, 1996). Snake venom disintegrins are small soluble proteins that bind with high affinity to the platelet integrin gpIIb/IIIa, thereby competitively inhibiting platelet aggregation (Gould *et al.*, 1990; Huang *et al.*, 1987, 1989; Kini and Evans, 1992; Musial *et al.*, 1990; Scarborough *et al.*, 1993). The homology between the disintegrin domain of the sperm protein fertilin and snake venom integrin ligands gave the first indication that fertilin may bind to an integrin on the egg. In addition to fertilin, several additional MDC proteins are expressed in the mammalian testis (Barker *et al.*, 1994; Heinlein *et al.*, 1994; Perry *et al.*, 1994; Wolfsberg and White, 1996), and MDC proteins have also been reported in several other tissues and cells (Emi *et al.*, 1993; Krätzschmar *et al.*, 1996; Perry *et al.*, 1992; Podbilewicz, 1996; Rooke *et al.*, 1996; Weskamp and Blobel, 1994; Weskamp *et al.*, 1996; Wolfsberg *et al.*, 1995; Yagami-Hiromasa *et al.*, 1995; Yoshida *et al.*, 1990).

In the frog *Rana pipiens*, sperm entry is thought to be restricted to the animal hemisphere of the egg (Elinson, 1975), suggesting the presence of a localized sperm receptor. However, we are not aware that a sperm receptor on frog eggs has been identified, or of a mechanism to explain the localized sperm–egg fusion, or of candidate frog sperm proteins with a role in fertilization. Here we report the identification of five distinct MDC cDNA sequence tags from the testis of *Xenopus laevis* through a PCR-based approach. A full-length cDNA clone for one of these proteins, xMDC16, was isolated and sequenced, and Northern blot analysis revealed that xMDC16 is expressed in the testis, but not in ovaries, eggs, or the somatic tissues examined (heart, liver, and muscle). Our finding that MDC family members exist in *X. laevis* testis suggested that they could also participate in *X. laevis* fertilization. Indeed, we found that peptides which mimic the predicted integrin-binding sequences of three of five xMDC proteins inhibited fertilization of *X. laevis* eggs, and certain substitutions in one of the inhibitory peptides (xMDC16) eliminated its ability to inhibit fertilization. These data indicate that amphibian fertilization, like mammalian fertilization, may involve one or several members of the MDC protein family.

METHODS

Generation of cDNA sequence tags for xMDC proteins by PCR. cDNA sequence tags encoding the partial disintegrin domain of xMDC9, 11.1, 11.2, 13, and 16 were isolated by PCR from *X. laevis* testis cDNA essentially as described (Krätzschmar *et al.*, 1996), using the following two degenerate primers: (a) 5′-GGI-GA(A/G)-IAI-TG(T/C)-GA(T/C)-TG(T/C)-GG-3′, derived from the conserved peptide sequence GEECDCG within the disintegrin domain and (b) 5′-GCA-G(T/A)I-(C/T)TC-IG(G/C)-(A/G)AI-(A/G)TC-(A/G)CA-3′, derived as an anti-sense primer from the conserved peptide sequence CDLPE(L/H)C. Comparison of the disintegrin domain of xMDC9, 11.1, 11.2, 13, and 16 with the disintegrin domain of other known MDC proteins and phylogenetic analysis were performed using the Megalign program (DNASTAR software, Clustal method, PAM Matrix 250).

Cloning and sequencing of xMDC16. The cDNA sequence tag for xMDC16 was used to screen, under high stringency conditions (Blobel *et al.*, 1992), a *X. laevis* testis cDNA library which we constructed according to the manufacturer's instructions (Stratagene ZAP cDNA Library Synthesis kit; see also Krätzschmar *et al.*, 1991). One cDNA clone with an insert of 2467 bp was picked and sequenced completely (Sequenase; USB, Cleveland, OH) in both orientations by a primer walk, with primers spaced approximately every 250 nucleotides. Analysis of the xMDC16 cDNA and translated protein sequence was performed using MacVector sequence analysis software (Kodak IBI, New Haven, CT).

Northern blot analysis. Total RNA was isolated from heart, liver, skeletal muscle, and ovaries of adult female *X. laevis*, from eggs laid as described below, and from the testis of male *X. laevis* (Chomczynski and Sacchi, 1987). Northern blot analysis was performed under high stringency as previously described (Weskamp and Blobel, 1994), using a random-primed ^{32}P-labeled xMDC16 cDNA as probe. Two separate blots were probed with the xMDC16 cDNA probe, one containing the RNA from testis, heart, muscle, and liver and the second containing RNA from testis (as a positive control), ovaries, and eggs. For each sample, 15 μg of total RNA was loaded per lane, and the integrity of the ribosomal RNA was monitored on a separate gel. As a control the samples were reprobed with a random-primed ^{32}P-labeled cDNA for *X. laevis* fibronectin (kindly supplied by Drs. D. Alfandari and D. DeSimone). Specifically, the blot containing RNA from testis, heart, muscle, and liver samples was reprobed with the control probe after removal of the xMDC16 probe (Fig. 3, left), whereas for the samples of RNA from testis, ovaries, and eggs, a second identical blot was prepared and probed with the control probe (Fig. 3, right).

Peptides. The peptides used in this study matched the predicted integrin-binding sequence of each xMDC protein identified, which corresponds to the three amino acid residues found in lieu of the snake venom RGD sequence. The peptides also contained five additional amino acid residues from the protein sequence, in-

cluding cysteines at each end in the case of cyclic peptides. Cyclic peptides were synthesized by the Memorial Sloan–Kettering microchemistry facility using an ABI 433 peptide synthesis apparatus in combination with Fmoc chemistry. The peptides were resuspended at a concentration of 1 mg/100 ml in a solution containing 5% DMSO (Fluka) and 0.01% TFA, and stirred for 6 hr at room temperature in a beaker. The resulting oxidation of the peptides leads predominantly to intramolecular disulfide bonds between the carboxy- and amino-terminal cysteine residue, although intermolecular disulfide bonds can also form, which would give rise to multimeric peptides. After oxidization, the peptides were lyophilized and resuspended in 0.1% TFA, and monomeric cyclic peptides were purified by reverse-phase HPLC on a C_{18} column (2.0 × 25 cm; Vidac), which allows separation of monomeric from multimeric peptides. The purified peptides were analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (PerSeptive Biosystems Voyager RP) to confirm the expected molecular weight and to rule out the presence of dimeric or oligomeric peptides. Linear peptides were supplied by Chiron mimotypes (Victoria, Australia) at a purity of greater than 95%.

Frog egg preparation and fertilization. Adult wild-type *X. laevis* were injected with human chorionic gonadotropin (300 to 400 U) 12 hr prior to obtaining eggs. Where needed, eggs were dejellied by shaking for <1 min in 0.3% β-mercaptoethanol in 1/3 modified Ringers (MR), pH 8.8, followed by washing with MR, pH 6.5, and MR and F1, pH 7.8 (F1 composition in m*M:* NaCl, 41.25; KCl, 1.75; Na_2HPO_4, 0.5; NaOH, 1.9; Hepes, 2.5; $CaCl_2$, 0.2 ; $MgCl_2$, 0.063; EGTA, 5.0. MR composition in m*M:* NaCl, 100; KCl, 1.8; $MgCl_2$, 1.0; $CaCl_2$, 2.0; Hepes, 5.0). For certain experiments, eggs were manually stripped of their vitelline coats using finely tipped watchmaker forceps. Eggs for all experiments were used within an hour of release from the female. Fertilization was scored as the occurrence of the first cleavage, since this was the most accurately measurable indicator of fertilization. A sperm suspension was obtained by mincing approximately 1/8 of a frog testis in F1. Dejellied eggs were fertilized with egg jelly water-treated sperm (1:1, egg jelly water:sperm). The treatment of *X. laevis* sperm with jelly water is thought to cause the acrosome reaction, as this treatment is necessary to allow the sperm to bind to and fuse with dejellied eggs. Egg jelly water was isolated by gently washing eggs in 1/3 MR (2.7 ml 1/3 MR per 1 g eggs) and separating loosened jelly coats by passing the eggs over a nylon mesh. A final concentration of 10% Ficoll was added and the mixture was subsequently frozen (J. H. Roberts and J. Gerhart, personal communication; see also Heasman *et al.*, 1991).

Peptide treatment of gametes. Between 5 and 10 jelly-intact or dejellied eggs were incubated in 200 or 400 μl of F1 in the presence of varying concentrations of peptide dissolved in F1 (0, 0.25, 0.5, and 1 m*M*), contained within agarose wells. After 15 to 60 min, the eggs were inseminated. In the case of the dejellied eggs, the sperm were incubated for 10 min with egg jelly water prior to insemination. Eggs were incubated, and insemination was carried out in agarose-coated petri dishes, which provide a jelly-like surface for sperm to swim along, as opposed to glass. For certain controls, sperm were preincubated with peptide, then washed twice with a 20-fold excess of F1 before addition to eggs that had not been exposed to peptide.

RESULTS

Isolation of Partial MDC cDNA Fragments by PCR from Testis

In order to test whether MDC proteins play a role in amphibian fertilization, we searched for MDC proteins that are expressed in the testis of the African clawed frog *X. laevis*, using a PCR-based strategy (Krätzschmar *et al.*, 1996). We identified 86 PCR products in the expected size range that encoded disintegrin domains of MDC proteins, and a total of five distinct PCR tags could be distinguished (Fig. 1A). Phylogenetic analysis using DNASTAR Megalign software indicated that four of the sequences may represent homologues of known mammalian proteins (Fig. 1B). These PCR sequence tags have therefore tentatively been assigned the MDC/ADAM number of their closest mammalian homologue (Huovila *et al.*, 1996). In Fig. 1A, the xMDC sequence tags are shown as part of an alignment including all of the disintegrin domain sequences that were used for the phylogenetic analysis. The sequence identity between the deduced protein sequence of the xMDC9 PCR sequence tag and mouse MDC9 is 60.4%, and 75% between xMDC11.1 and human MDC11, 59.6% between xMDC11.2 and human MDC11, and 74.5% between xMDC13 and mouse MDC13, also referred to as meltrin β (Yagami-Hiromasa *et al.*, 1995). The fifth cDNA sequence tag did not show a clear relationship to other known MDC proteins and was therefore termed xMDC16 to reflect that it is the 16th presently known MDC protein for which a full-length sequence is available (see below). Tissue Nothern blot analysis with each of the xMDC cDNAs using RNA isolated from *X. laevis* testis, muscle, heart, and liver showed that xMDC16 was expressed only in testis, and not in the other three tissues examined (see below). xMDC11.1 and xMDC11.2 were expressed in testis and less abundantly in heart and muscle, and xMDC9 and xMDC13 were expressed in testis, muscle, heart, and liver (H. Cai and C. P. Blobel, manuscript in preparation).

Isolation of Full-Length xMDC16 cDNA and Northern Blot Analysis

Because of its testis-specific expression pattern, the xMDC16 cDNA was cloned from a *X. laevis* testis cDNA library (see Methods). The longest xMDC16 cDNA clone thus isolated harbored an insert of 2467 bp, and was sequenced completely on both strands using a primer walk strategy. It contained an open reading frame encoding 706 amino acid residues (Fig. 2A), which includes seven consensus sequences for N-linked glycosylation. Not counting the predicted signal sequence, the calculated molecular weight of nonglycosylated xMDC16 is 77.37 kDa. Northern blot analysis with the full-length xMDC16 cDNA as a probe showed a sharp band of 2.8 kb and a slightly more diffuse band between 2.4 and 2.6 kb in testis, but no detectable expression in *X. laevis* heart, muscle, liver, eggs, or ovaries (Fig. 3).

xMDC16 contains a metalloprotease domain with the active site consensus HEXXH, and analysis of the hydrophilicity plot of xMDC16 shows a predicted transmembrane domain in a position similar to that in other MDC proteins (Fig. 2B). However, compared to other MDC proteins, xMDC16 has a very short cytoplasmic tail. In the region

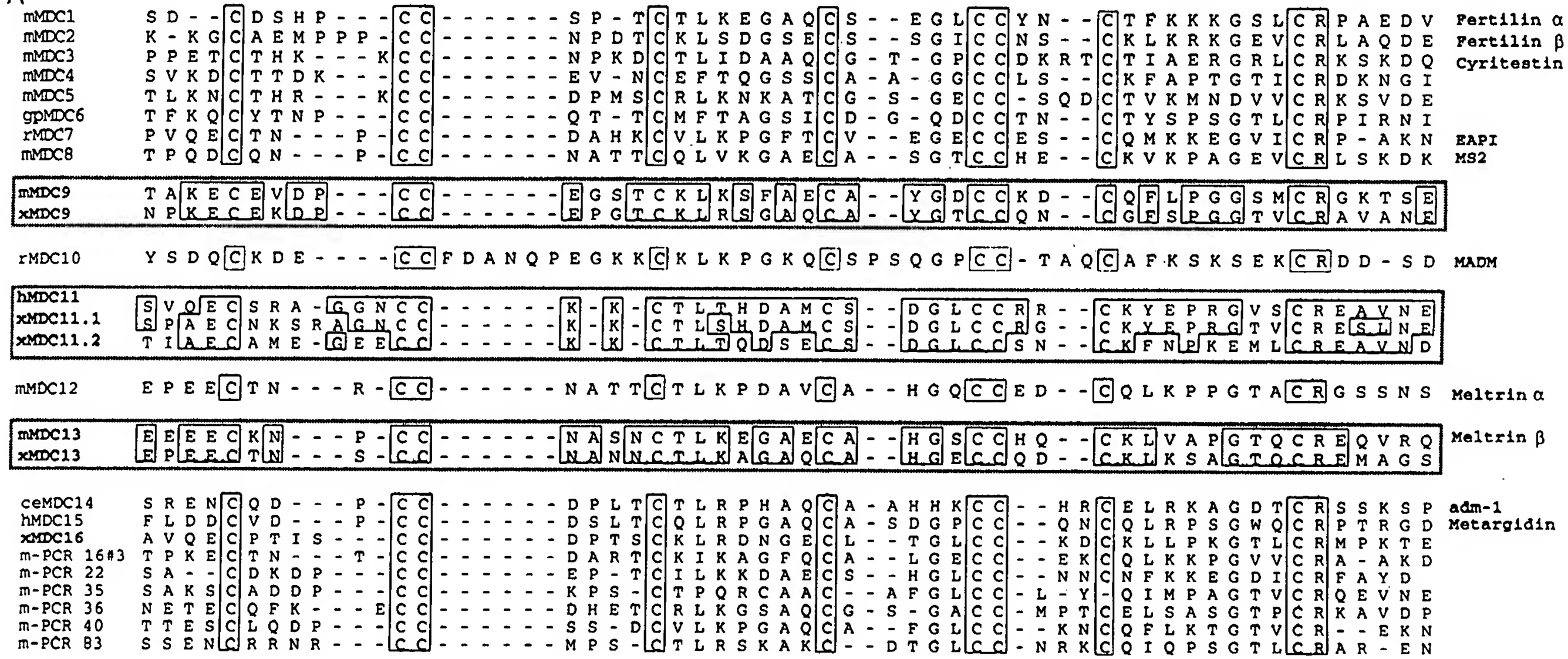

FIG. 1. Alignment and phylogenetic comparison of the disintegrin domains of xMDCs with other presently known MDC disintegrin domains. (A) The disintegrin domains of MDC proteins (1–16) are presented in order, with the numbers corresponding to those assigned to the identical ADAM proteins (Wolfsberg and White, 1996). For the new xMDC PCR sequence tags, the deduced protein sequence is shown in alignment with the closest known MDC protein, surrounded by a stippled box. The sequence identities between the disintegrin domain of xMDC proteins and their closest recognized homologue are, for hMDC9 and xMDC9, 60.4% (29 of 48 amino acid residues); for xMDC11.1 and hMDC11, 75% (36 of 48 amino acid residues); for xMDC11.2 and hMDC11, 59.6% (28 of 47 amino acid residues); and for xMDC13 and mMDC13, 74.5% (35 of 47 amino acid residues). Other names used for individual MDC proteins are shown in bold on the right. The six sequences labeled m-PCR represent mouse PCR sequence tags reported earlier (Weskamp and Blobel, 1994). These proteins will be assigned an MDC/ADAM number once the full-length sequence is available. The GenBank Accession Nos. for MDC 1-13 are reported in Wolfsberg and White (1996), and the GenBank Accession Nos. for other proteins in the alignment are as follows: U78187 for xMDC9, U78189 for xMDC11.1, U78188 for xMDC11.2, U78186 for xMDC13, U68185 for MDC14, U41767 for MDC15, and U78185 for xMDC16. (B) Phylogenetic relationship of the disintegrin domains shown in (A). All xMDCs are marked with an asterisk, and all mammalian MDC proteins which are known to be expressed in the testis are marked with an arrow.

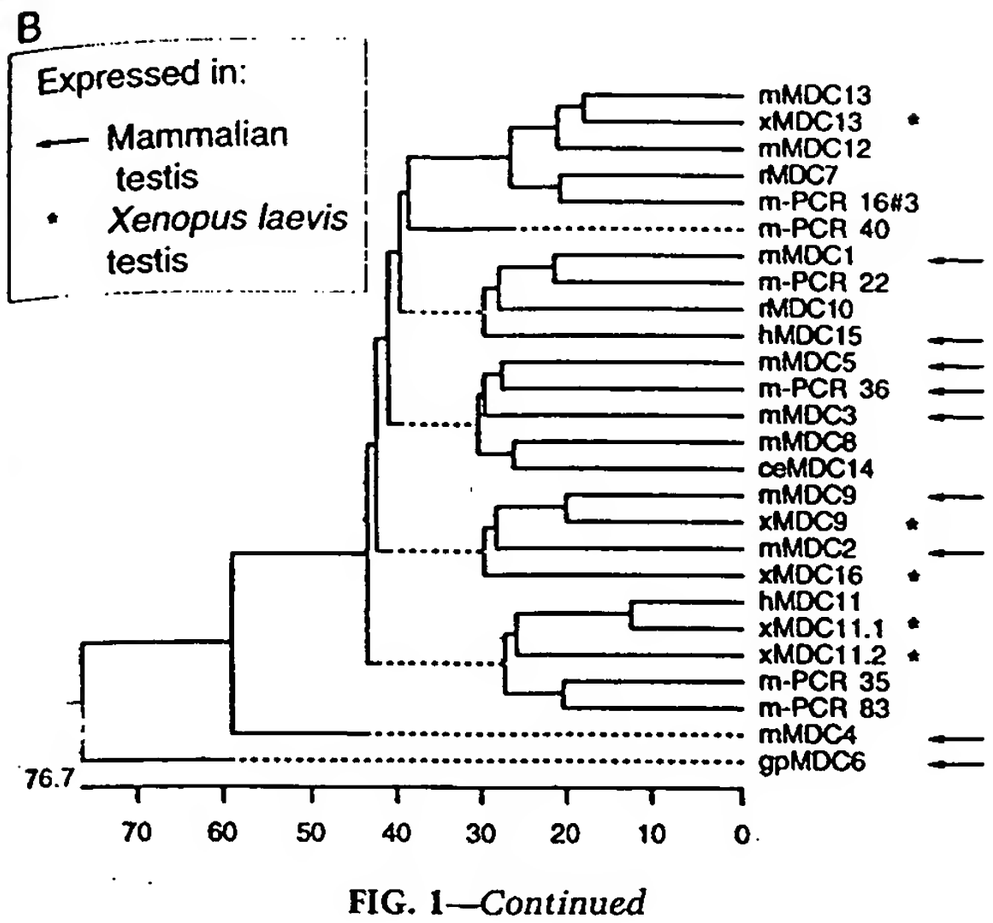

FIG. 1—*Continued*

where fertilin α and meltrin α have a predicted fusion peptide which can be modeled as an amphipathic helix with about 20 amino acid residues (Blobel *et al.*, 1992; Huovila *et al.*, 1996; White, 1992), xMDC16 instead harbors a cluster of only four hydrophobic amino acids, arguing against a role of this region as a fusion peptide. A longer hydrophobic stretch exists between amino acid residues 495 and 510 of xMDC16, the significance of which remains to be determined.

Fertilization Is Inhibited by Peptides Containing Predicted xMDC Integrin-Binding Sites

Guinea pig and mouse fertilization can be inhibited by peptides that mimic the predicted integrin-binding domain of fertilin β (Almeida *et al.*, 1995; Myles *et al.*, 1994). To test whether one or more of the xMDC proteins reported here may play a role in fertilization, we examined peptides that mimic the predicted integrin-binding site of xMDC9, xMDC11.1, xMDC11.2, xMDC13, and xMDC16 for their ability to inhibit fertilization of *X. laevis* eggs. Jellied eggs were preexposed for 15 to 60 min to 0.25 to 1 m*M* concentrations of certain peptides (Fig. 4A), prior to the addition of sperm. Fertilization was scored by counting eggs that underwent cleavage after incubation with *X. laevis* sperm. Incubation of jellied eggs with a 1 m*M* concentration of the peptides derived from xMDC9, xMDC13, and xMDC16 inhibited fertilization of *X. laevis* eggs by close to 80, 100, and 80%, respectively, compared to untreated eggs. The level of inhibition varied with the concentration of the xMDC9, xMDC13, and xMDC16 peptides, with half-maximal inhibition at a concentration between 0.5 and 0.25 m*M* (see Fig. 4A). The peptides mimicking the predicted binding sequence of xMDC11.1 and xMDC11.2 proteins had no effect on *X. laevis* fertilization at a concentration of 1 m*M*.

To confirm that the peptides were not interfering with binding to the jelly coat, jellied and dejellied eggs were incubated separately in the presence of different concentrations of xMDC9 and xMDC16 peptides (0.25, 0.5, and 1 m*M*). In the presence of 1 m*M* each specific peptide, jellied eggs were fertilized at rates of 8/35 (xMDC9) and 4/24 (xMDC16) compared to dejellied egg fertilization rates of 8/35 (xMDC9) and 6/35 (xMDC16), using eggs from three females for each peptide. Likewise, at the lower concentrations of 0.5 and 0.25 m*M*, no significant difference in inhibition of fertilization was seen between dejellied and jellied eggs, indicating that the peptides were not affecting a fertilization step involving the jelly coat. The protocol used here to remove the jelly coat does not strip the vitelline coat from the egg. Therefore, we wished to test whether the xMDC16 peptide was interfering with binding to the vitelline coat or was acting to inhibit interactions between the sperm and the egg plasma membrane. Vitelline coat-free eggs from three females were incubated with xMDC16 peptide (1 m*M*) and inseminated with jelly water-treated sperm. Only 4 of 24 eggs treated with the peptide underwent cortical contraction after fertilization, compared with 14 of 18 eggs not treated with peptide. Because eggs without the vitelline envelope tend to collapse, cleavage was not scored in these experiments. These data suggest that the xMDC16 peptides were competing for a sperm-binding site on the egg plasma membrane and not affecting a fertilization step involving the jelly coat or the vitelline envelope.

Since the fertilization assay uses zygote cleavage as indicator for fertilization, we wished to exclude that the peptides were interfering with a postfertilization event, including the first cleavage. Eggs incubated in the presence of 1 m*M* xMDC13 or xMDC16 peptide after insemination were not inhibited from cleaving (31/37 eggs from two females for xMDC16, 8/8 eggs from one female for xMDC13), indicating that the inhibitory effect observed was indeed on fertilization. In order to rule out the possibility that the peptides were acting on the sperm instead of the eggs, sperm were preincubated with peptide and extensively washed to remove unbound peptides before insemination of the eggs. Under these conditions, 59 of 63 eggs were fertilized with xMDC16 peptide-treated sperm, and 30 of 32 eggs were fertilized using xMDC9 peptide-treated sperm, compared to 77/77 eggs for untreated sperm (two males, four females).

One peptide, xMDC16, was selected for a more detailed analysis of the potential sequence requirements for inhibition. A scrambled peptide containing amino acid residues of the predicted integrin-binding site of xMDC16 in a different order (CRMPKTEC changed to CKEMTRPC) and two other control peptides in which the predicted binding sequence KTE was replaced with the sequence ATA or AAA all failed to block fertilization at concentrations of up to 1 m*M* (see Fig. 4B). However, a peptide in which the glutamate residue within the KTE motif had been replaced with an alanine to

A

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Signal Sequence                    Pro-domain
MMGFRLPFIV CHVVVVALSF NLNVTDWLDS YEIVTPLFIE DRTVNKTREL   50
TYAIMIDSKD YIIHLVHNPM LVTTDFKVFS YSKTYSLYFD EPHILTHCYY  100
QGYVQGYPNS VVAMNTCSGL RGIMHTDEFI YAIEPSEPLH HFEHIAYSME  150
                                   Metalloprotease domain
QFKTTIDCLL DTSIYLRSTE SSAIEQGLCV RNKTAYVEVF MVATQQQFEF  200
HHENRTFLLE SMLDLINSVN AVYQDLNTHI ILVGIEIWSN DNLINTEGRN  250
VHDILSDFLA WKKEYLDEHI KCAVAVLSLR DETIPSSGLS HLRGACSLDD  300
SAFMIMMNLE KPMLSLDLFV HELGHILGMR HTSGCKCKS GKPACVMASR  350
                                   Disintegrin domain
GLLSLGFSDC NEKDMEMFFA SSEASCLWKE LSQCGNNILE QGEKCDCGSV  400
QECPTISCCD PTSCKLRENG ECLTGLCCKD CKLLPKGTLC RMPKTECDLA  450
                                   Cysteine-rich domain
EYCDGASNHC PLDMYKQNGA ACNNGTSVCY ENRCYDYNKH CESIFGEGAT  500
VAPFSCFQWV NTIGDRFGNC NTEREMVECN IKNVMCGRLQ CQNVSSEMKP  550
VDSKTAIILT PNGNSWCWGL DFHSKHSSFD LGSVPDGAPC DTGKICLNKR  600
                EGF-like domain
CVSSSLLSYD CDAEHKCGGN GVCNNRKNCH FYSQWAPPNC TQSGYGGSVD  650
                                   TM domain          Cytoplasmic-
SGPITKHSPV EFMPEGGILR LYDTNAASLM VPFLMTTLYH TLVIECFQSI   domain 700
YLYTVWSN                                                706
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B

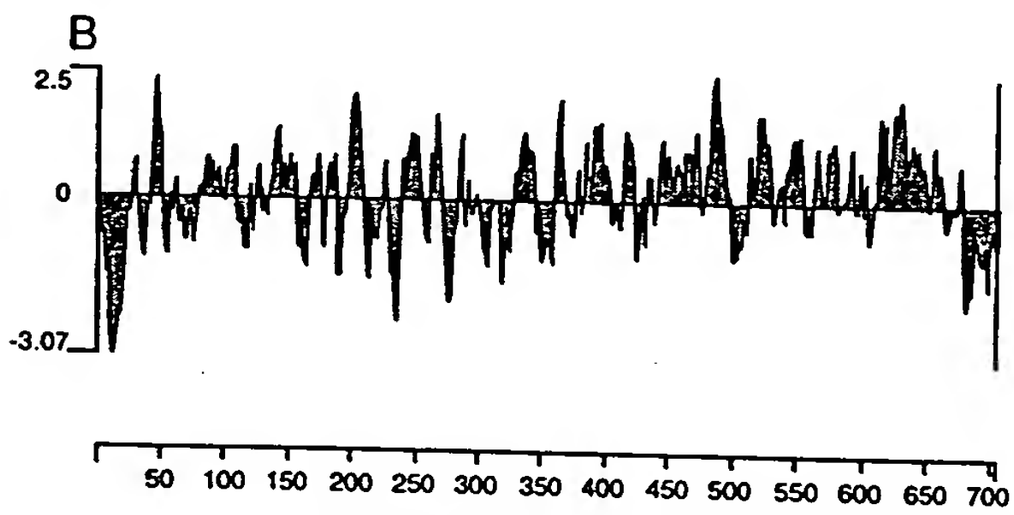

FIG. 2. Sequence and hydrophilicity plot of xMDC16. (A) The deduced protein sequence of xMDC16 is shown, with the predicted amino-terminus of each protein domain marked by a vertical bar. Consensus sequences for N-linked glycosylation sites are marked with an asterisk, the predicted metalloprotease consensus sequence is surrounded by a stippled rectangle, and the predicted integrin-binding sequence (KTEC), found in lieu of the RGD sequence in snake venom disintegrins, is surrounded by a bold rectangle. (B) A hydrophilicity plot of xMDC16 was generated using DNASTAR Protean software.

yield KTA inhibited fertilization as efficiently as the KTE peptide.

DISCUSSION

In this study, we report the isolation of five distinct cDNA sequence tags from the testis of *X. laevis* encoding MDC proteins that may play a role in *X. laevis* fertilization. Of these five cDNA sequence tags, xMDC16 was the only one not expressed in the somatic tissues examined here, and therefore could encode a testis-specific member of the family. Phylogenetic comparison of the deduced disintegrin domain sequence of xMDC proteins to the disintegrin sequences of other presently known MDC proteins revealed the closest homologies between xMDC9 and mouse and human MDC9, between xMDC11.1, xMDC11.2, and human MDC11, and between xMDC13 and mouse MDC13, which is also referred to as meltrin β (Yagami-Hiromasa *et al.*, 1995).

Because of its apparently testis-specific expression pattern, xMDC16 was cloned and sequenced. The deduced xMDC16 protein sequence contains all protein domains that are characteristic for MDC proteins (see above), including a metalloprotease domain with the catalytic site consensus sequence HEXXH. Although xMDC16 is more closely related to fertilin α (28.8% sequence similarity) than to any of the other xMDC proteins reported here, this relatively low degree of sequence similarity suggests that xMDC16 is not the fertilin α orthologue.

One of the questions about MDC proteins expressed in

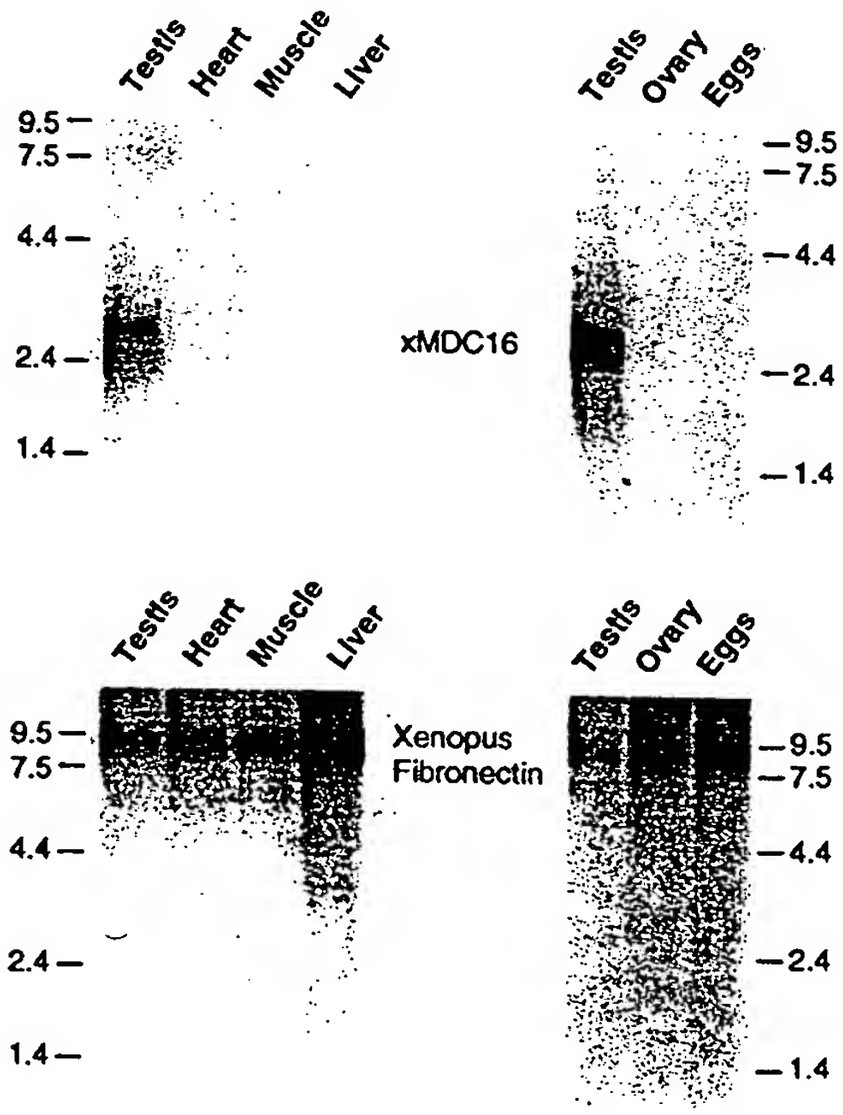

FIG. 3. Northern blot analysis of xMDC16. The top shows Northern blots of RNA isolated from the *X. laevis* tissues testis, heart, liver, and muscle (left) and testis, ovaries, and eggs (right). Both blots were probed separately with ^{32}P-labeled xMDC16 cDNA under high stringency conditions (see Methods). The lower left shows the same blot as the upper left probed as a control with ^{32}P-labeled *X. laevis* fibronectin cDNA (kindly provided by Drs. D. Alfandari and D. DeSimone), after removal of the xMDC16 probe. The lower right shows a Northern blot of RNA samples that were identical to those shown in the upper right (testis, ovary, and eggs), probed with the control ^{32}P-labeled *X. laevis* fibronectin cDNA.

testis (see Fig. 1B) is whether these are present on mature sperm and may thus play a role in fertilization. Even the more widely expressed MDC proteins may have a specific role in fertilization, since for example the widely expressed $\alpha 6\beta 1$ integrin is thought to play a specific role in fertilization as an egg receptor for sperm (Almeida *et al.*, 1995). To test for a potential role in *X. laevis* fertilization of the five xMDC proteins presented here, we performed peptide inhibition studies in a fashion similar to that previously described for fertilin β-derived peptides, which can inhibit guinea pig fertilization (Myles *et al.*, 1994) and mouse fertilization (Almeida *et al.*, 1995; Evans *et al.*, 1995). In the guinea pig and mouse inhibition studies, the inhibitory peptides were designed to mimic the predicted integrin-binding site of fertilin β, which is the sequence found in lieu of the RGD sequence in snake venom disintegrins (TDE in guinea pigs, QDE in mice) (Almeida *et al.*, 1995; Blobel *et al.*, 1992; Evans *et al.*, 1995; Myles *et al.*, 1994). We found that, of the five xMDC peptides tested, those corresponding to the predicted binding sequence of xMDC9, xMDC13, and xMDC16 were capable of inhibiting *X. laevis* fertilization in a concentration-dependent manner. For all three peptides half-maximal inhibition was achieved with peptide concentrations between 0.5 and 0.25 m*M*. The peptides corresponding to the predicted binding site of xMDC11.1 and xMDC11.2 did not affect fertilization, suggesting that these proteins may not play a direct role in fertilization. These data are consistent with a possible role for one or several of the newly identified xMDC proteins in fertilization. If the peptides indeed inhibit fertilization by binding to an egg integrin, then it will be interesting to determine whether several peptides bind to the same receptor or, alternatively, to different receptors.

The predicted integrin-binding sequence of xMDC16 was chosen to further define amino acid residues that are important for the inhibitory effect exerted by this peptide. At a concentration of up to 1 m*M*, a scrambled peptide, and two peptides in which the sequence KTE was changed into AAA or ATA, did not inhibit fertilization. This result suggests that the order of amino acids is important, and specifically that the lysine and glutamate residues of the predicted binding site are important for inhibition. However, a peptide in which only the glutamate residue had been replaced with alanine (KTE to KTA) inhibited fertilization as effectively as the KTE peptide. This result suggests that the acidic glutamate residue does not contribute significantly to the inhibitory function of the xMDC16 peptide. In contrast, in RGD-containing integrin ligands, the acidic aspartate residue is thought to bind to, and then possibly displace, a divalent cation in the integrin ligand-binding site (Bergelson and Hemler, 1995; Lee *et al.*, 1995). Mutagenesis of the RGD sequences, for example in the snake venom disintegrin kistrin, has shown that not only the acidic aspartate residue, but also the arginine residue, are critically important for integrin binding (Dennis *et al.*, 1993). In this context it is interesting to point out that the xMDC13 peptide, which inhibits fertilization as effectively as the xMDC16 peptide, also lacks an acidic amino acid residue in the predicted binding site.

In the fertilization inhibition studies described here, we used the first egg cleavage as an indicator that fertilization had occurred. In principle, inhibition of fertilization either could be due to competition of the peptide for a sperm-binding site on an egg receptor or could result from a partial activation of the egg, which would then prevent fertilization from occurring via an egg-induced block to polyspermy. However, since treatment of eggs with the peptides at the concentrations reported here ($\leq$1 m*M*) did not result in an observable cortical contraction or envelope elevation, we conclude that the inhibitiory effect was not due to activation of the eggs. In this context it is interesting to point out that RGD-containing peptides can trigger egg activation in *X. laevis* eggs (Iwao and Fujimura, 1996), although the exact mechanism of this activation remains to be determined.

Based on the peptide inhibition experiments described above, we propose that fertilization in *X. laevis* is a multistep process involving one or more MDC proteins on the sperm, which would bind to a single or several receptor(s) on the egg. The presence of several MDC proteins in mammalian testis (see arrows in Fig. 1B) would also be consistent with this idea. Fertilization could then be conceptually similar to the multistep process of leukocyte adhesion, in which different membrane-anchored integrin ligands (ICAM and VCAM) interact with leukocyte and endothelial integrins to promote leukocyte binding and extravasation (Springer, 1990, 1994). Since fertilization studies in mice have provided evidence that an integrin may function as a sperm receptor (Almeida *et al.*, 1995), the sperm receptor in *X. laevis* eggs may also be an integrin. Although integrins are expressed in *X. laevis* eggs (Gawantka *et al.*, 1992; Joos *et al.*, 1995; Müller *et al.*, 1993; Whittaker and DeSimone, 1993), it remains to be established which integrin subunits are expressed on the surface of mature *X. laevis* eggs. The $\beta 1$ integrin, for example, is apparently not present on the egg surface (Müller *et al.*, 1993), and the $\alpha 6$ integrin subunit, which has been implicated in mouse fertilization (Almeida *et al.*, 1995), cannot be detected in the *X. laevis* egg and embryo before neurulation (Lallier *et al.*, 1996). Nevertheless, in principle integrins are attractive candidate sperm receptor proteins since they can act as adhesion proteins and as signal-transducing receptors in cell–extracellular matrix and cell–cell interactions (Hynes, 1987, 1992; Ruoslahti, 1991).

In addition to a putative role as integrin ligands, MDC proteins with a metalloprotease consensus sequence (HEXXH) are also predicted to be functional metalloproteases, with a potential role in the release of membrane-anchored proteins, such as cytokines or cytokine receptors, or in proteolysis of the extracellular matrix (Basbaum and Werb, 1996). Since proteases can lead to egg activation in starfish (Carroll and Jaffe, 1995), it will be interesting to determine whether the metalloprotease domain of xMDC proteins may also have a specific function in fertilization and/or activation of the egg.

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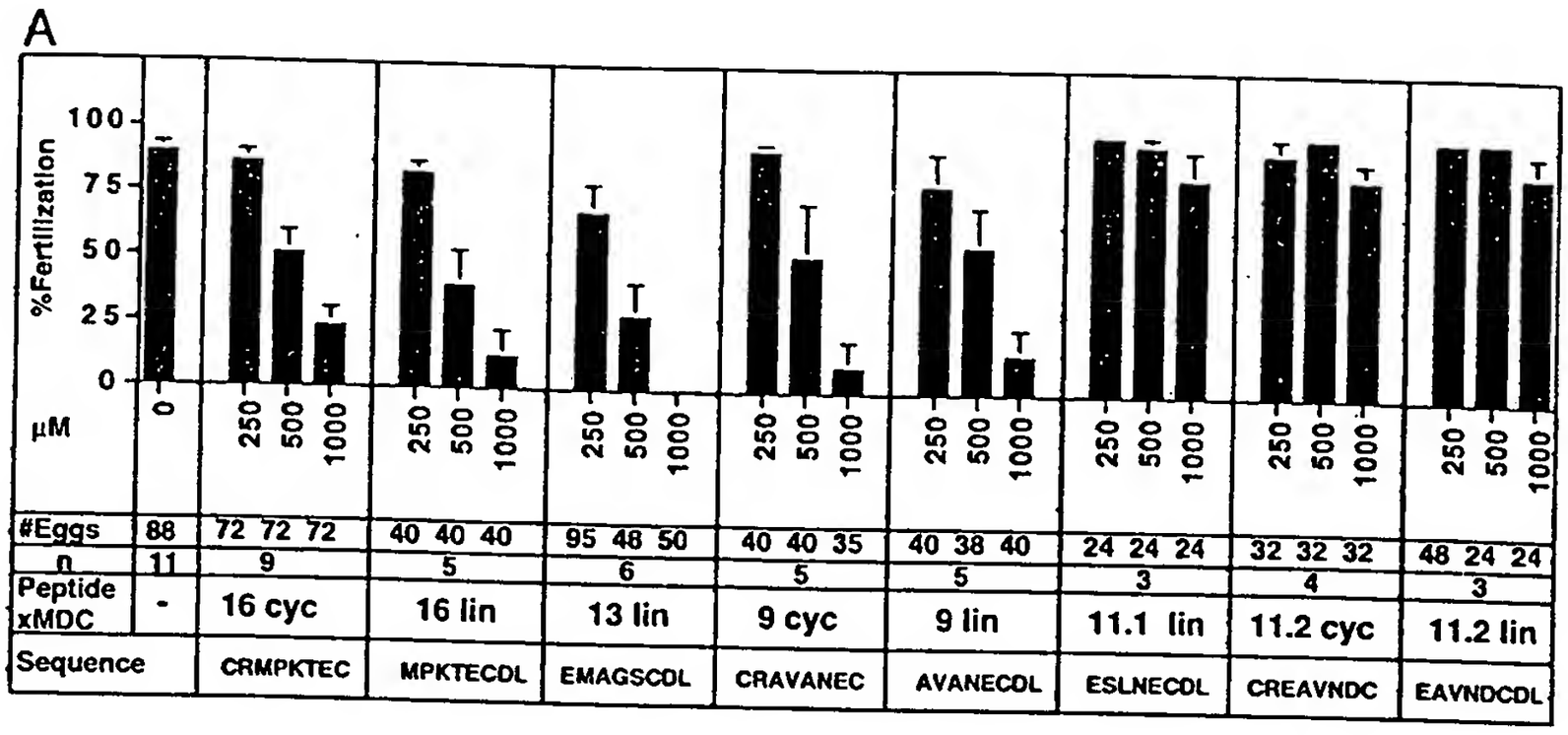

B

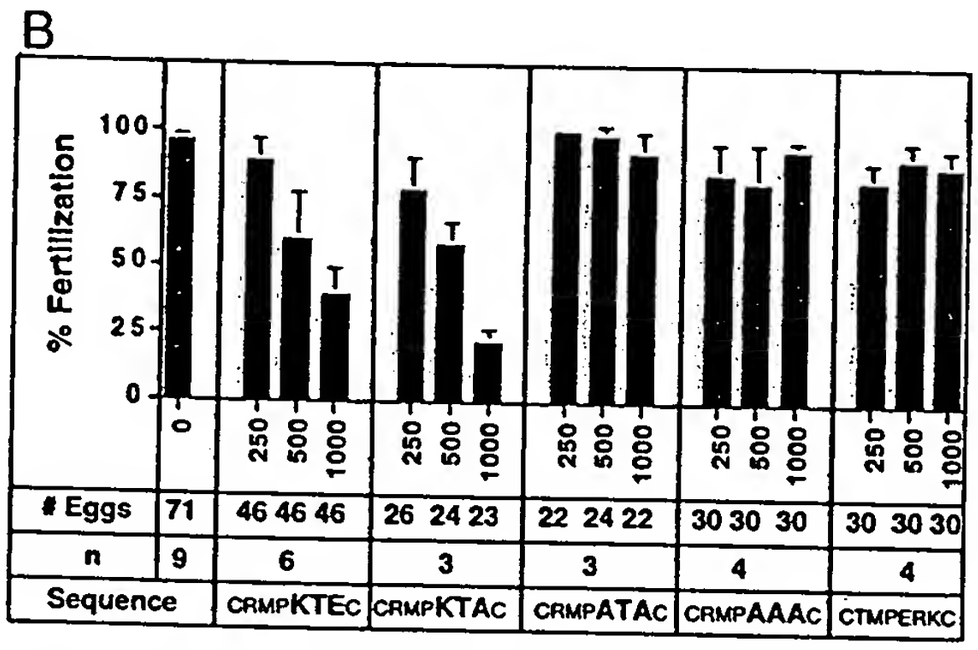

FIG. 4. Peptide inhibition of *Xenopus* fertilization. Jellied eggs were preincubated in the indicated different concentrations of peptides and then inseminated. The proportion of eggs that underwent the first cleavage were scored as fertilized. The error bars indicate the standard error of the mean among experiments. The number of experiments is indicated in the table (n), as is the total number of eggs used for different peptide concentrations. Eggs from a different female were used in each experiment, and the number of eggs per experiment was usually 8, although as few as 5 eggs or as many as 16 were scored in some instances. (A) Inhibition studies using cyclic (cyclized via disulfide bond formation, see Methods) and linear peptides corresponding to the predicted binding sequence of different xMDC proteins (see also Fig. 1A). The sequences of the peptides used here are shown in the table. The linear peptides include three carboxy-terminal amino acid residues that were determined from longer cDNA clones of the corresponding xMDC genes (H. Cai, J. Krätzschmar, and C. P. Blobel, manuscript in preparation). The first bar stands for 0 m*M* peptide and is the control for all of the peptide treatments. (B) Inhibition studies using variants of the xMDC16 peptides, in which individual amino acid residues of the predicted binding site were changed into alanine residues, and also the results obtained with a scrambled peptide. The number of eggs used for each concentration, the number of experiments for each peptide, and the sequences of the xMDC16 peptide variants are indicated.

In conclusion, we have identified five xMDC proteins that are expressed in the testis of *X. laevis*. Peptides corresponding to the predicted integrin-binding domain of three of five proteins inhibit fertilization, suggesting that xMDC proteins may play a role in *X. laevis* fertilization, in a fashion similar to that of mammalian MDC proteins like fertilin. Future studies will aim at a further biochemical and functional characterization of the role of different xMDC proteins in *X. laevis* fertilization. Using peptides and fusion proteins derived from xMDC16 and other xMDC proteins, it may be possible to isolate the putative receptor(s) for xMDC proteins on the egg surface and to use these proteins to advance our understanding of the mechanism of vertebrate sperm–egg membrane interactions.

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Function of Disintegrin-like/Cysteine-rich Domains of Atrolysin A

INHIBITION OF PLATELET AGGREGATION BY RECOMBINANT PROTEIN AND PEPTIDE ANTAGONISTS*

(Received for publication, August 19, 1996, and in revised form, March 11, 1997)

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Snake venom hemorrhagic metalloproteinase toxins that have metalloproteinase, disintegrin-like and cysteine-rich domains are significantly more potent than toxins with only a metalloproteinase domain. The disintegrin-like domains of these toxins differ from the disintegrin peptides found in crotalid and viperid venoms by the nature of their different disulfide bond structure and, in lieu of the disintegrins' signature Arg-Gly-Asp (RGD) integrin binding sequence, there is an *XXCD* disulfide-bonded cysteinyl sequence in that region. Due to these apparent differences, the contribution to the overall function of the hemorrhagic metalloproteinases by the disintegrin-like domain has been unknown. In this investigation we have expressed in insect cells the disintegrin-like/cysteine-rich (DC) domains of the *Crotalus atrox* hemorrhagic metalloproteinase atrolysin A and demonstrated that the recombinant protein (A/DC) can inhibit collagen- and ADP-stimulated platelet aggregation. Using synthetic peptides, we have evidence that the region of the disintegrin-like domain that is positionally analogous to the RGD loop of the disintegrins is the site responsible for inhibition of platelet aggregation. For these synthetic peptides to have significant inhibitory activity, the -RSECD- cysteinyl residue must be constrained by participation in a disulfide bond with another cysteinyl residue. The two acidic amino acids adjacent to the middle cysteinyl residue in these peptides are also important for biological activity. These studies emphasize a functional role for the disintegrin-like domain in toxins and suggest structural possibilities for the design of antagonists of platelet aggregation.

The distinctive characteristic of envenomation by a crotalid or viperid snake is local and in severe cases systemic hemorrhage. The profuse hemorrhage observed is usually due to the synergistic action of a large number of toxins in the venom (1, 2). However, the toxins primarily responsible for hemorrhage are snake venom zinc metalloproteinases (SVMPs),[1] which are members of the reprolysin subfamily of the M12 family of metalloproteinases (3–5). These toxins, as isolated from crude venom, belong to one of three related structural classes, P-I, -II, and -III, which primarily differ from one another by the presence of additional domains on the carboxyl side of the metalloproteinase domain (4). The P-I class has only a metalloproteinase domain, whereas the P-II class has a disintegrin or disintegrin-like domain carboxyl to the proteinase domain. The P-III class metalloproteinases have yet another domain, the cysteine-rich domain, which is found carboxyl to the disintegrin-like domain.

The P-III class of venom metalloproteinases is related to the ADAMs/MDCs group of type I integral membrane protein. These protein groups have homologous proteinase, disintegrin-like and cysteine-rich domain structures, and these proteinases are classified as members of the reprolysin subfamily of metalloproteinases (5). However, the ADAMs/MDC proteinases possess additional carboxyl-terminal structures comprised of epidermal growth factor-like, transmembrane, and cytoplasmic domains (6–9).

The biological function of many of the ADAMs/MDCs proteins is unclear, except for the fertilins α and β, which are involved in egg-sperm fusion (10), meltrins, which are involved in myoblast fusion (11), and KUZ, a *Drosophila* protein which participates in neurogenesis (12). In the case of the fertilins, which are the most functionally characterized of the group, the disintegrin-like domain of fertilin is thought to modulate egg-sperm fusion by interaction of the disintegrin-like domain of the sperm fertilin with the $\alpha_6\beta_1$ integrin on the egg (13). The structural features of the disintegrin-like domain important in this interaction are not known with certainty but may involve a specific sequence of the disintegrin-like domain of fertilin (13).

Disintegrins are peptides, isolated from the venoms of crotalid and viperid snakes, and range in length from 49 to 84 amino acid residues. They function as potent inhibitors of platelet aggregation (14–16). The RGD sequence in a 13-residue β-loop structure (the RGD loop) is the critical structural moiety responsible for biological activity and is central to the interaction of the disintegrins with the platelet integrin $\alpha_{IIb}\beta_3$ (17, 18). The disulfide bond structure of these peptides also contributes to the activity of the disintegrins (19, 20). The disintegrins are derived from homologous precursors of the P-II class of snake venom metalloproteinases by the processing of precursors comprised of pre-, pro-, metalloproteinase, and disintegrin domains (21).

The homologous region of the class P-III SVMPs differs from the disintegrins and their P-II precursors in several ways. Due to these differences, we have termed them "disintegrin-like" domains. Disintegrin-like domains have two additional cysteinyl residues compared with the disintegrins, and therefore the disulfide bond arrangement is likely to be different. We hypothesize that one of these cysteines is involved in a disulfide bond

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[1] The abbreviations used are: SVMPs, snake venom metalloproteinases; Acm, acetoamidomethyl; AcNMPV: *A. californica* nuclear polyhedrosis virus; ADAMs, a disintegrin-like and metalloproteinase proteins; A/DC, recombinant disintegrin-like/cysteine-rich protein from atrolysin A; MALD-TOF, matrix assisted laser desorption-time of flight; MDCs, metalloproteinase disintegrin-like cysteine-rich proteins; PAGE, polyacrylamide gel electrophoresis.

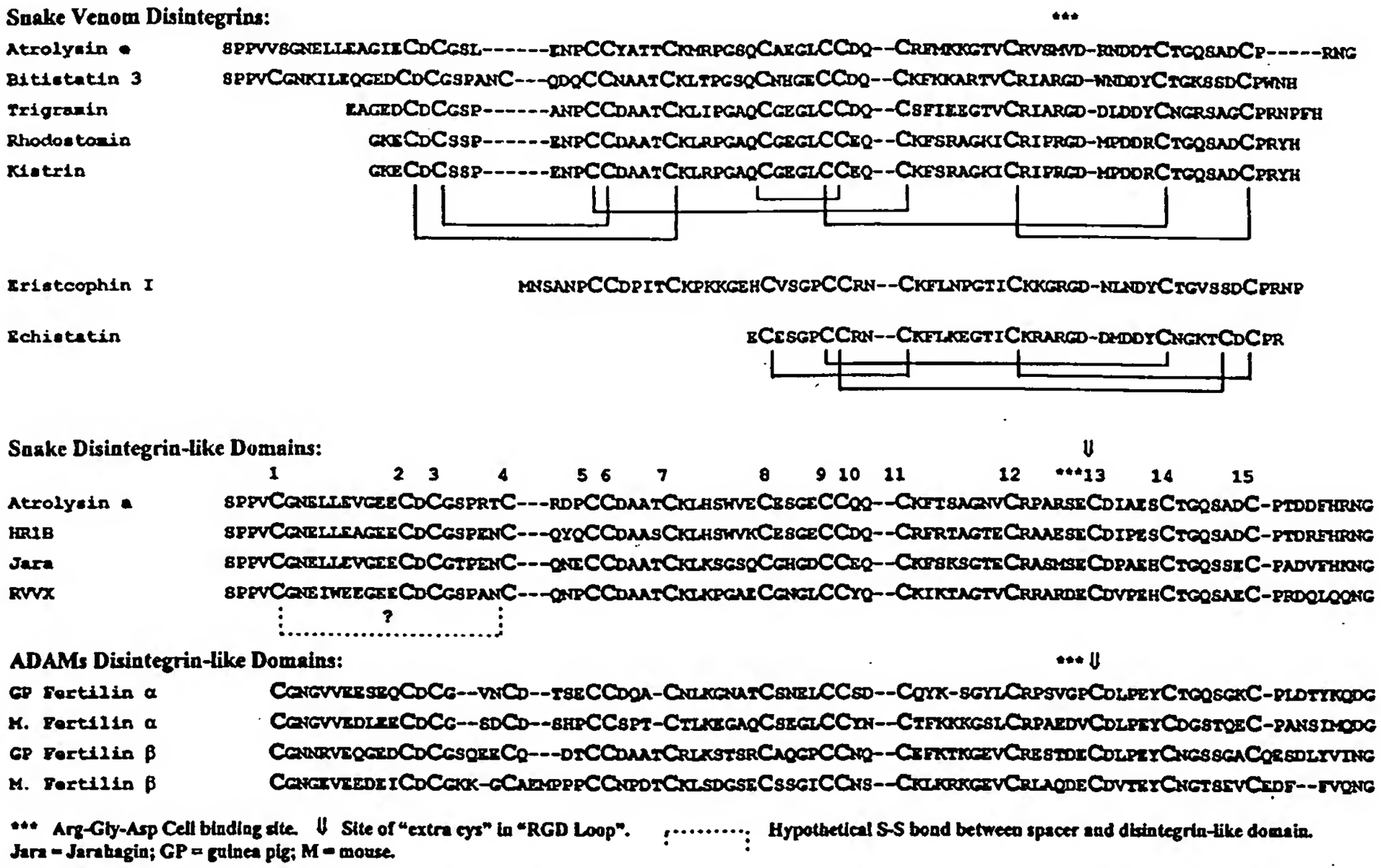


FIG. 1. **Comparison of the structure of the disintegrins with the non-RGD disintegrin-like domains of the SVMPs and the ADAMs.**

with a region amino-terminal to the disintegrin-like domain (the spacer region) which links the disintegrin-like domain with the proteinase domain (Fig. 1). The other cysteinyl residue is thought to be in a disulfide bond linkage with a cysteinyl residue in the cysteine-rich domain carboxyl to the disintegrin-like domain (22). This would form the spacer region, the disintegrin-like domain, and cysteine-rich domain into one continuous, disulfide bond-interconnected structure. The other notable difference between the disintegrin-like domains of the SVMPs and the disintegrin peptides is that while most disintegrins contain the RGD integrin-binding consensus sequence, to date no disintegrin-like domain of the class P-III SVMPs has been reported with the RGD consensus sequence. Furthermore, the additional cysteinyl residue found in the *XX*CD sequence described above for disintegrin-like domains lies in the middle of the loop where in the disintegrins the RGD sequence is located. Therefore, the topology of the disintegrin-like domain in this area is probably very different from that observed in the disintegrins proper (23, 24). This would certainly be the case if that cysteinyl residue were involved in a disulfide bond.

Since the class P-III hemorrhagic proteinases are significantly more potent than the class P-I toxins, we hypothesize that the additional carboxyl domains in the P-III toxins make an important contribution to the overall higher potency of this class of hemorrhagic toxins (22). To explore this concept, we have expressed in insect cells the combined disintegrin-like/cysteine-rich domains (A/DC) of atrolysin A, the most potent hemorrhagic toxin from the western diamondback rattlesnake *Crotalus atrox*. We now report on the ability of the recombinant A/DC protein as well as synthetic peptides designed from the SECD sequence region of the disintegrin-like domain to inhibit platelet aggregation. The structural role of the middle cysteinyl residue and adjacent acidic residues in the SECD loop region of disintegrin-like domains is also described.

EXPERIMENTAL PROCEDURES

Cloning of Atrolysin A Disintegrin-like/Cysteine-rich Domains (A/DC)—Standard recombinant DNA techniques were used to clone the A/DC fragment into the baculovirus pMbac vector (Stratagene Cloning Systems, La Jolla, CA). The DNA fragment encoding the disintegrin-like and cysteine-rich domains of atrolysin A was generated by polymerase chain reaction from an atrolysin A cDNA clone (4). Two oligonucleotide primers were designed for the polymerase chain reaction amplification and cloning. The upstream primer was 5′-CAATGACCCGGGGCAAACAGATATAATTTCAC-3′ and the downstream primer used was 5′-GATCTGGATCCTCAAATCTGAGAGAAGCCAGA-3′. These two primers were designed to include *Sma*I and *Bam*HI restriction sites, respectively, for in-frame insertion into the *SmaI/Bam*HI-linearized pMbac vector. The pMbac vector contains the signal sequence for melittin so that the recombinant protein should be secreted into the media. Prior to ligation into the pMbac vector, the A/DC polymerase chain reaction fragment (657 base pairs) was ligated into the pCR II TA cloning vector (Invitrogen) for propagation and then restriction with *SmaI/Bam*HI. The accuracy and frame information of A/DC in the pMbac vector was confirmed by complete DNA sequence and restriction analysis of the insert (25). Recombinant baculoviruses were generated by co-transfection of Sf9 (*Spodoptera frugiperda*) cells with the pMbac vector containing the A/DC insert (pMbac/A/DC) and AcNMPV nuclear polyhedrosis virus according to manufacturer's instructions (BaculoGold Transfection Kit, PharMingen). Plaque assays were performed, and following three rounds of plaque purification a population of homogeneous, recombinant A/DC baculovirus particles were obtained.

Expression and Isolation of Recombinant A/DC from Sf9 Insect Cells—Sf9 cells at a cell density of 2–3 × 10^{-6} cell/ml were transfected with the recombinant baculovirus at a ratio of 10 plaque-forming units per insect cell. The infected cells were harvested after 4 days by centrifugation at 4 °C for 15 min at 3000 rpm. The pelleted cells were resuspended in lysis buffer (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) and then disrupted in a French press. The suspension was centrifuged at 4 °C for 30 min at 15,000 × *g* and the resultant supernatant dialyzed against 20 mM Tris-HCl buffer, pH 8.0 at 4 °C. The dialyzed solution was then loaded onto a DEAE-cellulose ion-exchange column (1.5 × 40 cm) equilibrated with dialysate buffer, and the column was developed with a linear gradient of 0–1 M NaCl in the equilibration buffer. Fractions were monitored by SDS-

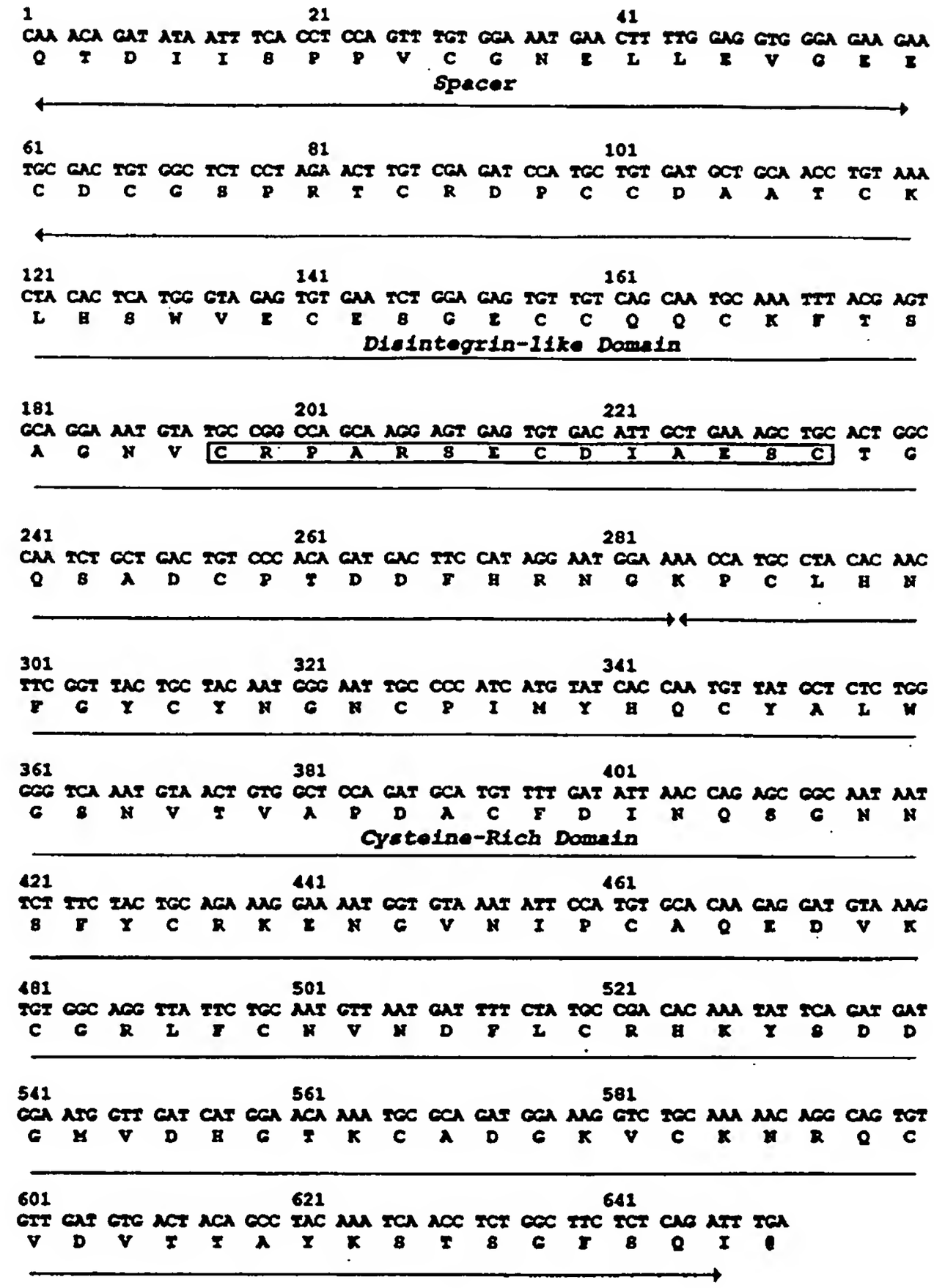


FIG. 2. **cDNA sequence and virtual protein sequence of atrolysin A/DC.** The recombinant protein sequence QTD- begins at the start spacer region. The RGD-like loop region is *boxed*.

PAGE on 12% gels (26) and by Western blot analysis using an anti-atrolysin A polyclonal antibody (27). The fractions identified as containing A/DC were pooled and dialyzed against the standard equilibration buffer. This dialyzed solution was then applied to a MonoQ 5/5 column (Pharmacia Biotech Inc.), and the column was developed with a 0–1 M NaCl gradient in the equilibration buffer. Pooled fractions containing A/DC were concentrated using a Centricon-30 concentrator (Amicon) and then loaded onto a Sephacryl S200 (Pharmacia) column (1.5 × 150 cm) developed with a 20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, at a flow rate of 15 ml/h. Fractions containing the A/DC protein, which were homogeneous by SDS-PAGE and Western blot analysis following this chromatography, were pooled, concentrated with a Centricon-30 cartridge, and stored at −20 °C.

Characterization of Recombinant A/DC—Isolated A/DC protein was subjected to amino-terminal sequence analysis on an ABI 470A protein sequencer operated according to manufacturer's instructions. The molecular mass of A/DC was determined by MALD-TOF mass spectrometry using a Finnigan Lasermat 1000 mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix. Atrolysin A and recombinant A/DC were alkylated under nonreducing conditions with [^{14}C]iodoacetate (53 mCi/mmol, DuPont NEN) in a 6 M guanidine HCl, 100 mM Tris-HCl, pH 7.5, alkylation buffer. Following alkylation, the proteins were desalted by reverse-phase chromatography on a C-8 (5 μm) column (5 × 30 mm) with a two-buffer gradient elution (buffer A, 0.1% trifluoroacetic acid in H_2O and buffer B, 0.1% trifluoroacetic acid in 80% $CNCH_3$ and 20% H_2O). Fractions containing ^{14}C-labeled A/DC were detected by absorbance at 214 nm, SDS-PAGE/autoradiography and by scintillation counting. Another *C. atrox* hemorrhagic metalloproteinase, atrolysin E, which has been demonstrated to contain a free cysteinyl residue (28), was subjected to the same alkylation procedure for use as a positive control.

Peptide Synthesis—All peptides were synthesized at the 50-μmol scale on a Symphony multiple peptide synthesizer (Rainin) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry as suggested by the manufacturer and modified according to individual peptide sequences (29). Peptide amide linker resin with 0.37 mmol/g substitution (PerSeptive Biosystems) was used as the solid support. Cleavage and deprotection of the peptides were performed on the synthesizer using 88% trifluoroacetic acid, 2% diisopropylsilane, 5% H_2O, and 5% phenol. Following cleavage, the ether-extracted crude peptide product was desalted and purified on a preparative C18 reverse-phase column (250 × 21.4 mm, Rainin) using a 0.1% trifluoroacetic acid/H_2O buffer with a gradient in acetonitrile (5–80%). The purity of all peptides was assessed by analytical reverse phase-high performance liquid chromatography and MALD-TOF mass spectrometry using previously established methods (29). Only peptides deemed greater than 98% pure by reverse phase-high performance liquid chromatography were used in the platelet aggregation studies. Following synthesis, all peptides were lyophilized and stored under N_2 at −30 °C until used.

In peptide 1 (see Table I) the carboxyl-terminal cysteinyl residue was protected with an Acm group, and the cysteinyl residues at positions 1 and 8 were protected with trityl groups that were removed during cleavage of the peptide from the resin. A disulfide from Cys^1 to Cys^8 was introduced by air oxidation. Similarly, peptide 2 was synthesized by protection of cysteinyls 8 and 14 with trityl groups and cysteinyl 1 with an Acm group.

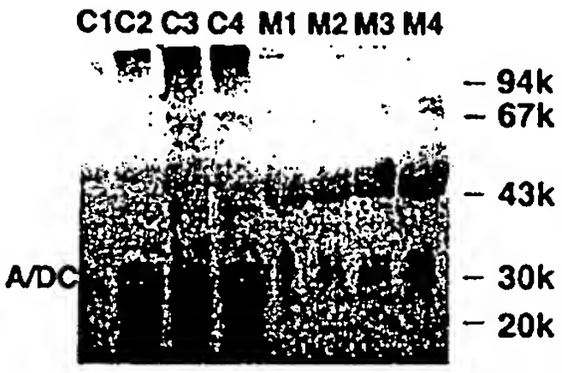


FIG. 3. **Western blot analysis of the time course of A/DC expression observed in Sf9 cells and secreted into the media.** *C1, C2, C3,* and *C4* and *M1, M2, M3,* and *M4* represent the relative amounts of A/DC found in lysed Sf9 cells and secreted into the media on days 1–4, respectively, following transfection. As can be seen, little or no specific or nonspecific background staining by anti-A/DC was observed at day 1 post-transfection (*C1*).

Peptide 3 was synthesized by protection of Cys^1 and Cys^{14} with Acm groups and Cys^8 with a trityl group. Following cleavage and deprotection, Cys^6 was alkylated with iodoacetic acid (30). Peptide 4, is based on the peptide 3 sequence except that the cysteinyl residue at position 8 is substituted by serine. Peptide 5 is based on peptide 1 structure except that the glutamyl residue at position 7 is substituted with an alanyl residue. Peptide 6 is also based on peptide 1 structure except the aspartyl residue at position 9 is substituted with an alanyl residue. Peptide 7 is the double alanyl-substituted peptide at positions 7 and 9. Peptide 8 is the Arg-Gly-Asp-Ala-substituted form of Arg-Ser-Glu-Cys sequence in peptide 3.

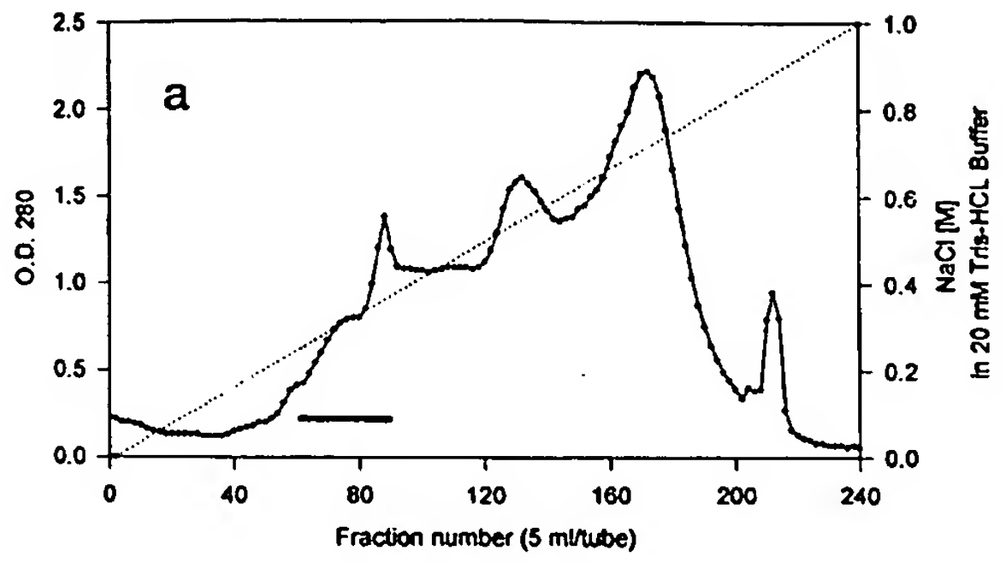
Platelet Aggregation Assays—Human blood was obtained from healthy donors who had not taken any medications within the previous 10 days. Blood was drawn into Becton Dickinson VACUTANER 228 containing 0.129 M sodium citrate with a final ratio of buffer to blood of 1:9. The tube was then centrifuged at 500 × *g* for 5 min. The platelet-rich plasma was transferred into a clean tube. Platelet concentration was measured with a Cell-Dyn-3000 cell counter (Abbott Diagnostics). The assay for platelet aggregation was conducted at 37 °C in an aggregometer (Payton, CO). The concentration of platelets used in each assay was 250,000 cells/μl at a final assay volume of 0.5 ml. The extent of platelet aggregation was quantitated by measuring the total amplitude at a predetermined time interval following addition of the platelet stimulant (collagen, 0.5 μg/ml, or ADP, 1 μM; ChronoLog Corp.). To assay for the ability of atrolysin A, the recombinant protein A/DC or the synthetic peptides, to inhibit platelet aggregation, the antagonists were dissolved in phosphate-buffered saline at pH 7.4, 20 mM $MgCl_2$ immediately before use. The antagonist solution was preincubated with platelet-rich plasma for 4 min at 37 °C prior to stimulation of platelet aggregation by collagen or ADP. The extent of the inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by the control dose of agonist (1 μM ADP or 0.5 μg/ml collagen) and then expressed as a percentage. IC_{50} values were determined from dose-response curves generated from the various concentrations used for the antagonists. All experiments were performed in triplicate on blood from at least three different donors.

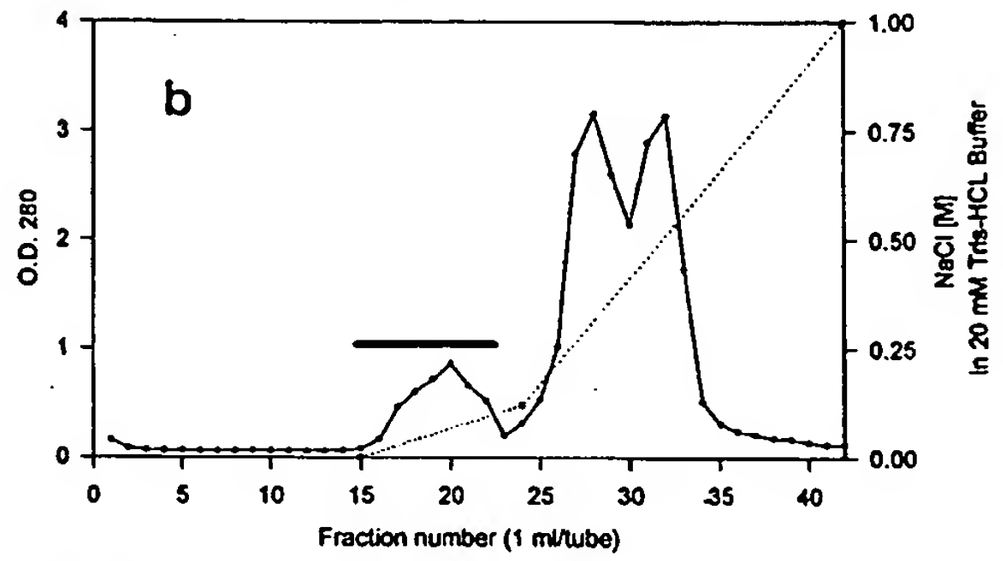
RESULTS

Expression, Isolation, and Characterization of Atrolysin A/DC—The translated DNA sequence for A/DC that was inserted into the pMbac vector is shown in Fig. 2. The expression of recombinant A/DC in transfected Sf9 cells was followed over time, and it was determined by Western blot analysis of Sf9 cells that day 4 post-transfected cells yielded the most product (Fig. 3). Little product was observed secreted into the medium even though a secretion expression vector had been used.

The chromatograms seen in Fig. 4 represent a typical isolation of A/DC from a 1-liter culture of Sf9 cells (2×10^6 cells/ml). From a 1-liter culture of transfected cells, the typical yield of purified A/DC was approximately 1 mg. The homogeneity of purified A/DC following the chromatography is seen in Fig. 5. Purified atrolysin A (4) was used as a standard for molecular weight comparison and Western blot analysis.

The amino-terminal sequence of A/DC determined from Edman degradation is given in Fig. 6. The first five residues are derived from the signal sequence of melittin, as coded for by the





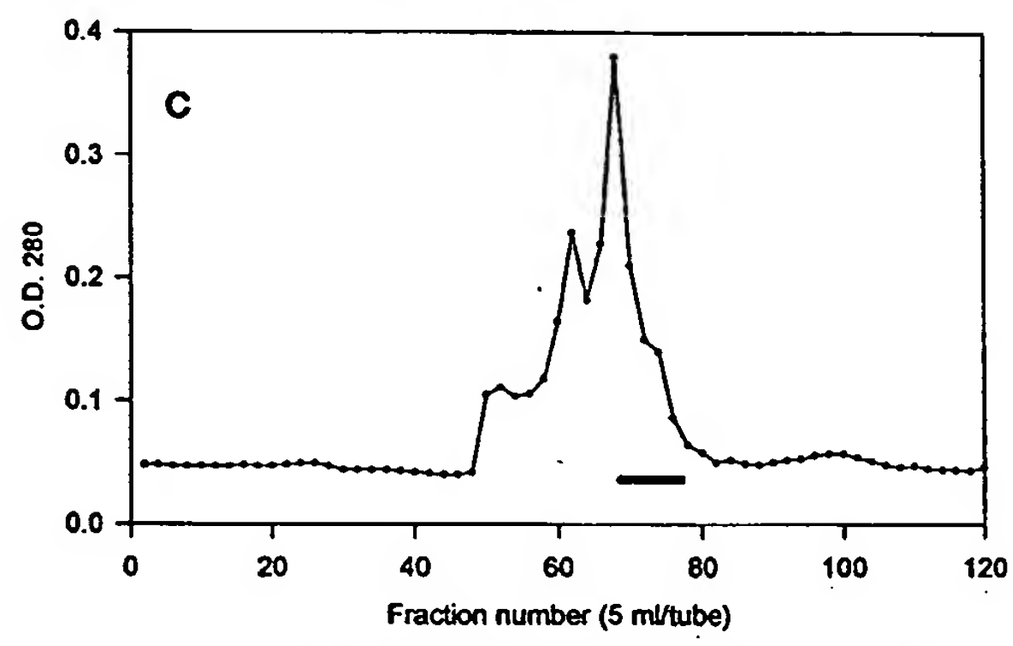


FIG. 4. **Isolation of recombinant A/DC.** *a,* DEAE-52 ion-exchange chromatography. The *dotted line* is the absorbance at 280 nm of the eluent, and the *dashed line* indicates the salt gradient. The *bar* shows the fractions where the A/DC protein was pooled. *b,* MonoQ 5/5 chromatography of the DEAE pool. The *dotted line* is the absorbance of the eluent at 280 nm, and the *dashed line* shows the elution of the salt gradient. The *bar* shows the fractions where the A/DC protein was pooled. *c,* Sephacryl S-200 gel filtration of the MonoQ pool. The *dotted line* is the absorbance of the eluent at 280 nm. The *bar* shows the fractions where A/DC was pooled.

expression vector, and are then followed by the A/DC sequence. The MALD-TOF mass spectrum of A/DC is also shown in Fig. 6. The experimental mass was determined to be 24,479 (M^+H ion) compared with 24,154 for the calculated mass, a difference of approximately 300 mass units or 1.3%. The explanation for this small discrepancy is unclear; however, there is a possibility for glycosylation of this protein. From protein sequence studies of atrolysin A, there are two *N*-linked glycosylation consensus sequences in the cysteine-rich domain, GSNVT and SGNNS (Fig. 2 and Ref. 4). Deglycosylation of A/DC with *N*-glycanase (Genzyme) caused a decrease in mass of approximately 278 mass units (data not shown) suggesting that glycosylation at one or both of those sites gives rise to the difference in the experimental *versus* the calculated mass of A/DC. The peak at 12314 mass units is the $M^{2+}H$ ion.

^{14}C-Carboxymethylation of nonreduced A/DC yielded product with no incorporation of label. Atrolysin E, which is known to have one "free" cysteine, incorporated approximately 26 mCi of ^{14}C per mmol, which relates to 0.8 cysteines/molecule of atrolysin E. This was similar to the 30 mCi of ^{14}C per mmol found in atrolysin A. The complete sequence of mature atrolysin A also has an odd number of cysteinyl residues (4). Since both atrolysin E and atrolysin A were approximately equally labeled, we concluded that atrolysin A has a single free cysteine. Furthermore, based on the lack of counts associated with the alkylation of A/DC, it was therefore concluded that all the cysteinyl residues of recombinant A/DC are involved in disulfide bonds and that the unpaired cysteinyl residue in atrolysin A resides in the metalloproteinase domain.

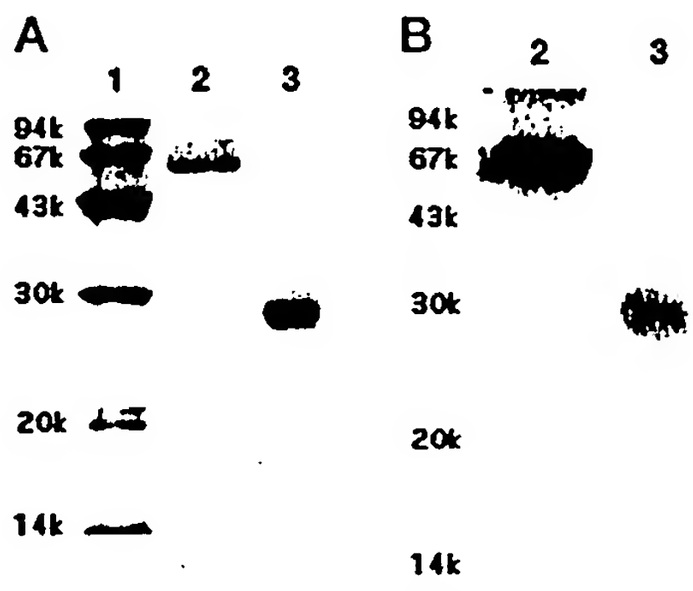


FIG. 5. **SDS-PAGE and Western blot of purified recombinant A/DC.** *A*, Coomassie Brilliant Blue-stained 12% SDS-PAGE of the pooled A/DC from the Sephacryl S-200 column. *Lane 1*, molecular mass markers; *lane 2*, atrolysin A; *lane 3*, recombinant A/DC. *B*, Western blot of identical gel. *Lane 2*, atrolysin A; *lane 3*, recombinant A/DC. Atrolysin A was purified from *C. atrox* venom according to the method of Bjarnason and Tu (45).

Platelet Aggregation Inhibition by Atrolysin A and A/DC—As seen in Figs. 7*A* and 8*A* and Table I, the hemorrhagic metalloproteinase atrolysin A was a potent inhibitor of collagen and ADP-stimulated platelet aggregation. The recombinant A/DC protein was also a potent inhibitor of platelet aggregation (Figs. 7*B* and 8*B* and Table I) but not to the same extent as atrolysin A, having a 4.3-fold greater IC_{50} value than atrolysin A for collagen-stimulated platelets. The IC_{50} of A/DC was of the same order of magnitude as observed for the disintegrins. Reduction and carboxyamidomethylation of A/DC caused a loss of all inhibitory activity (data not shown).

Platelet Aggregation Inhibition by Synthetic Peptides—The IC_{50} values for inhibition of collagen-stimulated platelet aggregation by synthetic peptides are seen in Table I. Peptide 1, which is the cyclized Cys^1-Cys^8 disintegrin-like region was a potent inhibitor of collagen-stimulated platelet aggregation with an IC_{50} of 218 ± 42 μM (Fig. 9). Peptide 2, which was the Cys^8-Cys^{14} form of the peptide, also was demonstrated to be an inhibitor of platelet aggregation with an IC_{50} of 391 ± 31 μM. Peptide 3 which is a linear peptide where the Cys^8 is alkylated and hence not constrained in a disulfide bond lacks inhibitory activity. Similarly, peptide 4, in which the Cys^8 of peptide 3 is substituted by a serinyl residue, also lacks activity. Peptides 5, 6, and 7 represent an alanine scanning substitution for the Glu^7 and Asp^9 residues adjacent to Cys^8. In peptide 5, which has a Glu^7 → Ala substitution, there is an approximate 2.5 decrease in activity compared with peptide 1. Peptide 6, which has an Asp^9 → Ala substitution, the IC_{50} value is approximately 1000 μM. When both the Glu^7 and Asp^9 are substituted with alanyl residues there is an even greater decrease in activity, with an apparent IC_{50} value significantly greater than 1 mM; however, due to peptide solubility, a more precise value

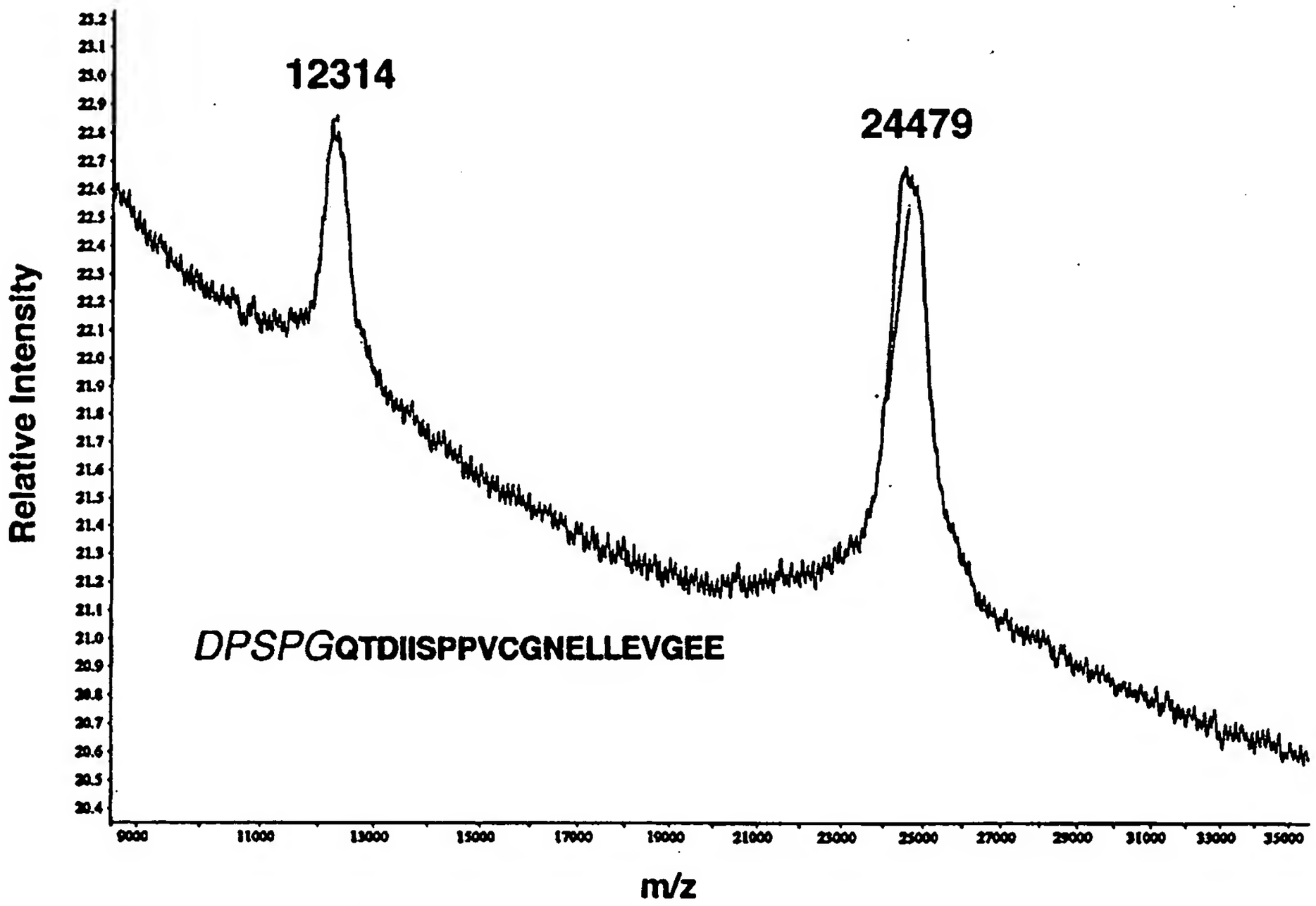


FIG. 6. **MALD-TOF mass spectrum of recombinant A/DC.** The amino terminus of recombinant A/DC as determined by Edman degradation is shown in the *inset*. The first five amino acids (*in italic*) are part of the melittin signal peptide.

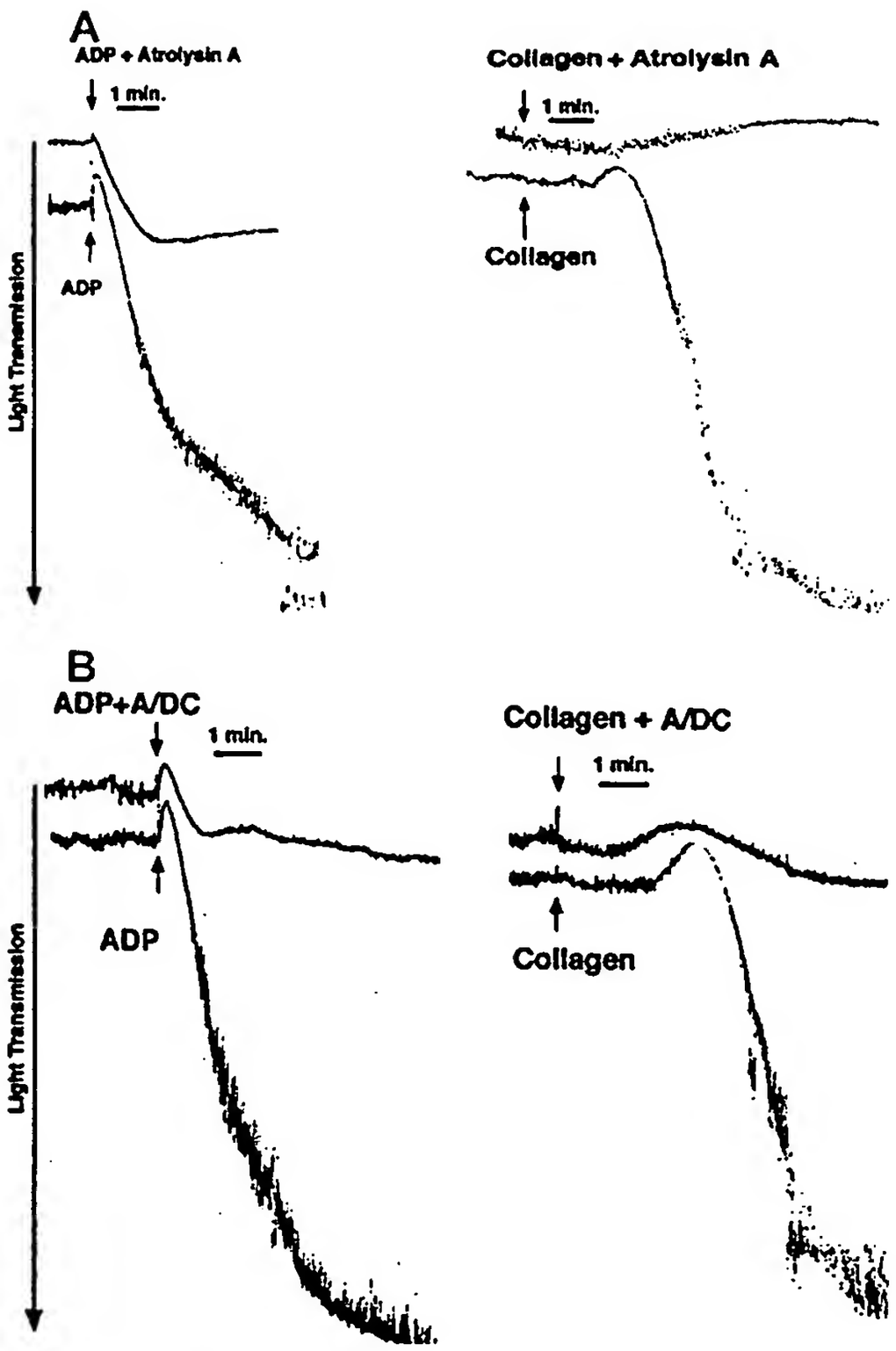


FIG. 7. **Effect of atrolysin A on human platelet aggregation.** *A*, aggregometer tracings of ADP-induced (2 μM) and collagen I-induced (0.5 μg/ml) human platelet aggregation inhibited by atrolysin A (0.3 μM). *B*, aggregometer tracing of ADP-induced (2 μM) and collagen I-induced (0.5 μg/ml) human platelet aggregation inhibited by A/DC (1.0 μM).

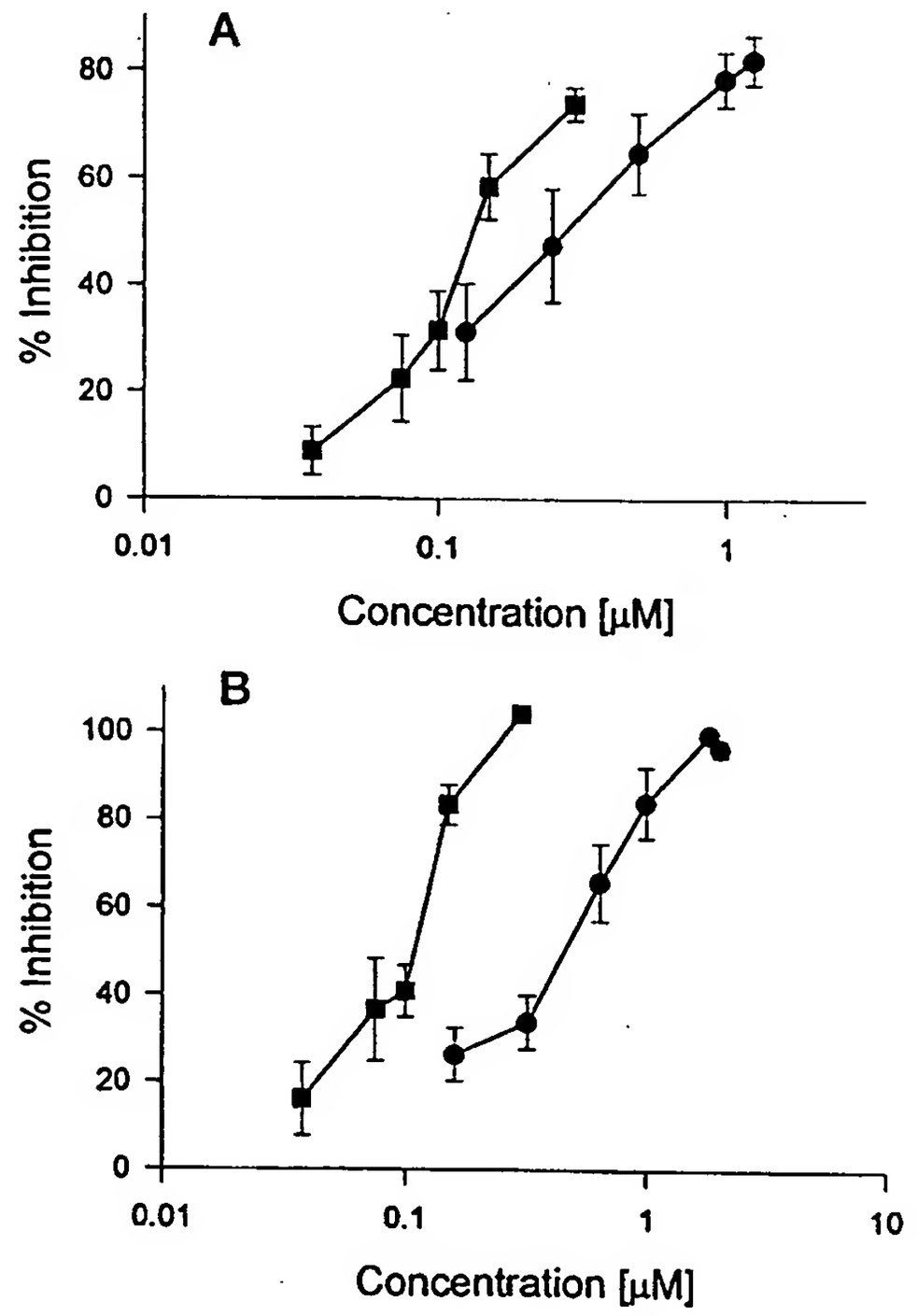


FIG. 8. **Concentration dependence of atrolysin A and A/DC inhibition of ADP-induced (2 μM) (*A*) or collagen I-induced (0.5 μg/ml) (*B*) human platelet aggregation.** Each *curve* represents the mean of 3–6 individual experiments. The *squares* represent the atrolysin A data and the *circles* A/DC.

could not be obtained. Peptide 8, which is the RGDA-substituted form of the RSEC residues in peptide **1**, was demonstrated to have potent collagen and ADP-stimulated platelet aggregation inhibition activity. The IC_{50} values for both collagen and ADP-stimulated platelet aggregation inhibition by peptide **1** are of the same order of magnitude for those observed with linear RGD containing peptides (14).

The protein inhibitors of platelet aggregation, such as atrolysin A, the recombinant A/DC protein, and the disintegrins themselves are significantly more active than their RGD/RGD-like loop peptide derivatives. Therefore, upon consideration of these data, it may be concluded that additional structural features other than those represented simply by a linear array of amino acids in the synthetic peptides, even though they are somewhat structurally constrained, are essential for the full inhibitory potential of these proteins.

DISCUSSION

It is generally observed that the higher molecular weight hemorrhagic metalloproteinases from snake venoms, as represented by the class P-III toxins, are significantly more toxic than the P-I class toxins (1). For example, the minimum hemorrhagic dose of atrolysin E, the most potent class P-II hemorrhagic toxin from the western diamondback rattlesnake *C. atrox*, is 1 μg/mouse. Atrolysin A, also from *C. atrox* venom, is a P-III toxin, which in addition to its proteinase domain has disintegrin-like and cysteine-rich domains. Its minimum hemorrhagic dose is 0.04 μg, making atrolysin A 25 times more hemorrhagic than atrolysin E, which in its mature form contains only a metalloproteinase domain. This observation led to our hypothesis that the additional domains of the P-III toxins contribute to their greater hemorrhagic potency. The functionality explored in this study is whether the disintegrin-like domain of the P-III toxins can serve to inhibit platelet aggregation and thus potentiate the production of hemorrhage.

The ability of atrolysin A to inhibit platelet aggregation is an interesting and novel observation stemming from this study. Unfortunately, due to the presence of three domains in atrolysin A, each with its own potential biological activity, it is unclear which domain(s) is responsible for the inhibition of platelet aggregation. The investigation into the contribution of the disintegrin-like domain of atrolysin A to inhibit platelet aggregation required expression of that recombinant domain. Expression of the disintegrin-like domain of atrolysin A failed to produce monomer product; therefore, we constructed and expressed in insect cells a recombinant protein comprised of the spacer region/disintegrin-like and the cysteine-rich domains of atrolysin A. The failure to express the disintegrin-like domain alone may be attributed to the possibility that there is one disulfide bond linking the spacer region with the disintegrin-like domain and one disulfide bond linking the disintegrin-like domain with a cysteinyl residue in the cysteine-rich domain (Fig. 2 and Ref. 22). This hypothesis is based on the

TABLE I

Inhibition of platelet aggregation by recombinant A/DC and synthetic peptides

NI, no inhibition; Acm, acetoamidomethylated cysteine; Ac, acetylated amino terminus; ND, not determined. Number in parentheses is the number of different donors used to generate data. S.E., standard error of the mean.

	Inhibitor	Platelet stimulant	
		Collagen (0.5 μg/ml)	ADP (1 μM)
		IC_{50} [μM] ± S.E.	
Atrolysin A		0.11 ± 0.01 (4)	0.24 ± 0.16 (3)
Recombinant A/DC		0.47 ± 0.05 (6)	0.32 ± 0.06 (4)
Peptide			
1	Ac-CRPARSECDIAESC(Acm)-NH_2	218 ± 42 (6)	ND
2	Ac-C(Acm)RPARSECDIAESC-NH_2	391 ± 31 (3)	ND
3	Ac-C(Acm)RPARSEC(cm)DIAESC(Acm)-NH_2	NI	NI
4	Ac-C(Acm)RPARSESDIAESC(Acm)-NH_2	NI	NI
5	Ac-CRPARSACDIAESC(Acm)-NH_2	546 ± 146 (3)	ND
6	Ac-CRPARSECAIAESC(Acm)-NH_2	~1000 (3)	ND
7	Ac-CRPARSACAIAESC(Acm)-NH_2	>>1000 (3)	ND
8	Ac-C(Acm)RPARGDADIAESC(Acm)-NH_2	156 ± 34 (3)	65 ± 3 (3)

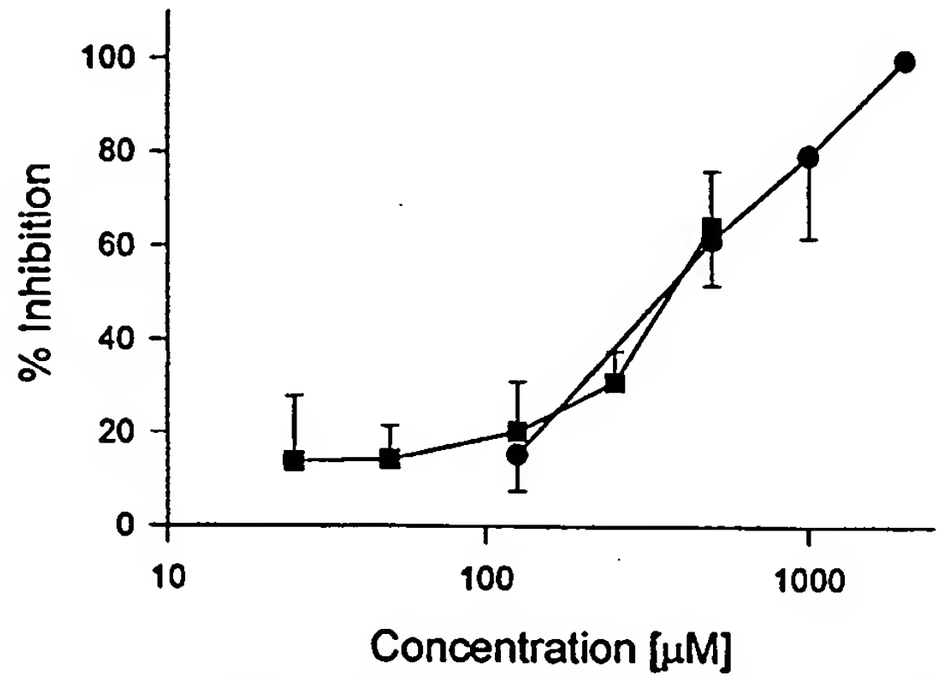


FIG. 9. **Concentration dependence of cyclized synthetic RGD-like loop peptides on collagen I (0.5 μg/ml)-induced human platelet aggregation.** *Circles* represent peptide 1, CRPARSECDIAESC(Acm) and *squares* represent peptide 2, (Acm)CRPARSECDIAESC.

comparison of the structures of disintegrin-like domains of the SMVPs and the ADAMs groups of the reprolysins to the structures of various venom disintegrins (4, 8, 14, 22, 31, 32). Ultimately, determination of the disulfide bond arrangement in the disintegrin-like and cysteine-rich domains of the P-III class of snake venom metalloproteinases will be necessary to prove the hypothesis.

Baculovirus expression of recombinant A/DC in insect cells was successful, ultimately yielding a protein with the ability to inhibit both collagen- and ADP-stimulated platelet aggregation. This suggests that A/DC is acting at the level of the $\alpha_2\beta_1$ collagen integrin and/or the fibrinogen receptor, $\alpha_{IIb}\beta_3$, on platelets (33–35). From the studies with the synthetic peptides one may conclude that the functional portion of A/DC involved in platelet binding resides in the disintegrin-like domain. However, at this point we have no direct evidence to exclude interactions with platelet integrins through the cysteine-rich domain of A/DC. The hemorrhagic toxin jararhagin, which is a structural homologue of atrolysin A, has been demonstrated to bind to the α_2 subunit of the $\alpha_2\beta_1$ integrin to inhibit platelet adhesion to collagen (36). Jararhagin has also been shown to cause the proteolytic loss of the platelet collagen receptor, $\alpha_2\beta_1$, and to degrade the adhesive plasma protein von Willebrand factor (37).

Although A/DC blocked platelet aggregation, it was somewhat less potent than atrolysin A. Whether this reflects the presence of additional inhibitory motifs in the structure of atrolysin A or the proteolytic effects of the metalloproteinase domain of atrolysin A, as appears to be the case with jararhagin, is unknown. Alternatively, the recombinant structure may not reflect the actual structure of the disintegrin-like domain as found in the P-III toxins. IC_{50} values for ADP-stimulated platelet aggregation inhibition by snake venom disintegrins range from approximately 100 to 555 nM (14, 16). The IC_{50} values of atrolysin A and recombinant A/DC for ADP-stimulated platelet aggregation inhibition determined in this study were 240 and 320 nM, respectively, which are comparable to those observed for the disintegrins. This similarity of potency of A/DC to the disintegrins is of great interest given the significant differences in the sequences in this region of the disintegrin (-like) domains of these proteins, particularly in the sense that A/DC lacks the cell-binding RGD consensus sequence. However, it has been reported that the RGD sequence need not be strictly conserved in the disintegrins for a potent ability to inhibit platelet aggregation (38, 39). In the case of barbourin, the disintegrin isolated from *Sistrurus miliarus barbouri*, in lieu of the RGD sequence, there is a conserved substitution of the arginine with lysine (39). This disintegrin has an IC_{50} value for inhibition of platelet aggregation similar to that observed for RGD-containing disintegrins (14). In another example, using a murine Fab fragment specific for the integrin $\alpha_{IIb}\beta_3$, Kunicki and colleagues (40) demonstrated that the cognate RGD sequence could be exchanged with RYD without an alteration in integrin recognition. These data suggest that some limited diversity in this sequence region may be tolerated and still give rise to a ligand with reasonable potency for inhibiting aggregation.

From structural studies of several disintegrins, the RGD sequence is found positioned within an extended, flexible β-loop structure where there is only limited conformational restriction of the RGD sequence (24, 32, 41, 42). Unfortunately, no similar structural information is available for disintegrin-like domain containing proteins. Reduction and alkylation of disintegrins cause a significant loss of platelet aggregation inhibition activity (19, 20), which is also the case with A/DC. Therefore, as in the disintegrins, the constrained display in this region in the disintegrin-like domain of A/DC is critical for activity. Never-

theless, there remain structural differences between these regions of the disintegrin-like domain and the disintegrins based on their differences in sequence, disulfide bonding patterns, and biological activities.

Using synthetic peptides, we have shown that the SECD sequence region in the disintegrin-like domain of A/DC is involved in platelet aggregation inhibitory activity. This region is the positional homologue of the RGD loop of the disintegrins. The two significant differences between this region of the disintegrin-like domains of the SVMPs and the RGD region of the disintegrins are the *XX*(E/*D*)CD substitution for RGD*XX* sequences observed in the disintegrins and the presence of a disulfide bonded cysteinyl residue (SECD) in the disintegrin-like domain region. Given these significant differences, it is very interesting that A/DC should have any ability to inhibit platelet aggregation and suggests a somewhat different interaction of this region of the disintegrin-like domain with the platelet $\alpha_{IIb}\beta_3$ integrin compared with that for the RGD disintegrins.

We have shown that the RSECD cysteinyl residue in atrolysin A and the recombinant A/DC is constrained by a disulfide bond. Although the synthetic peptides we tested were disulfide bonded from Cys^1 to Cys^8 or Cys^8 to Cys^{14}, this does not suggest that it is the same bonding pattern that occurs in the protein. However, since the RSECD cysteinyl residue is disulfide bonded in atrolysin A and A/DC, that region must be conformationally constrained with a quite different topology compared with the 13-member RGD loop of the disintegrins. This structural difference of the *XX*CD region of the disintegrin-like domain appears to be crucial for activity since the synthetic, linear peptides, which lack disulfide bonds or a free sulfhydryl, did not inhibit platelet aggregation.

A protein containing a disintegrin-like domain, which lacks a metalloproteinase domain, has been isolated and characterized from *Bothrops jararaca* venom and has a similar structure to the recombinant A/DC protein constructed for this study. This protein, jararhagin-C, begins with an amino-terminal sequence homologous to the spacer region of the P-III toxins followed by disintegrin-like and cysteine-rich sequences (43). Jararhagin-C can inhibit ADP- and collagen-induced platelet aggregation. The sequence of jararhagin-C is identical to the spacer/disintegrin-like/cysteine-rich domains of jararhagin, a 55-kDa hemorrhagic toxin from *B. jararaca* (44). Therefore, jararhagin-C is a proteolytically processed form of jararhagin. In this study, we have demonstrated that atrolysin A and the recombinant protein A/DC, comprised of the spacer/disintegrin-like/cysteine-rich domains, have potent platelet aggregation inhibitory activities. These data suggest an increased complexity for the venom of crotalid snakes from the standpoint of hemorrhagic toxicity. The high molecular weight P-III toxins can cause hemorrhage by direct proteolytic disruption of capillary basement membranes (31). They may also synergize hemorrhage production by inhibiting platelet aggregation via their disintegrin-like domain and by proteolysis of integrins and plasma adhesive proteins with their metalloproteinase domain. Indeed, the disintegrin-like and cysteine-rich domains may participate in the proteolytic specificity of these P-III toxins by directing them to the appropriate substrates. Hemorrhagic toxins are present in crotalid venoms, and their proteolytically processed fragments are comprised of spacer/disintegrin-like/cysteine-rich domains, as well as the disintegrins proper, all of which give rise to a very potent mixture of active proteins contributing to hemorrhage production and inhibition of platelet aggregation. Therefore, it is understandable that hemorrhage production is one of the foremost pathological consequences of crotalid snake envenomation.

In summary we have shown that the class P-III hemorrhagic toxin, atrolysin A from *C. atrox*, is a potent inhibitor of platelet aggregation. Our studies with synthetic peptides demonstrate that the region of the disintegrin-like domain of atrolysin A which is positionally analogous to the RGD loop of the disintegrins is primarily responsible for blocking platelet aggregation. The RSECD sequence in the disintegrin-like domain requires conformational restriction through disulfide bonding of the cysteinyl residue for biological activity. These considerations, plus the fact that the critical sequence is not an RGD sequence, suggest that different structural parameters are responsible for the biological activity of the disintegrin-like domains of the P-III toxins and the disintegrin-like domains of the ADAMs/MDC proteins. The data reported in this study provide a new structural framework for the development of novel integrin antagonists.

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